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IN VITRO AND IN VIVO MEASUREMENT OF PERCUTANEOUS PENETRATION OF LOW MOLECULAR
WEIGHT TOXINS OF MILITARY INTEREST
ANNUAL REPORT

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B. W. Kemppainen; M. Mehta; R. G. Stafford;
R. T. Riley; C. R. Clark

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The purpose of this contract is to : (i) use an <u>in vitro</u> system to assess the ability of low molecular weight toxins to penetrate through human and guinea pig skin, (ii) validate the results obtained in the <u>in vitro</u> system by conducting comparative studies of <u>in vitro</u> and <u>in vivo</u> cutaneous penetration in guinea pigs, (iii) assess the effects of specific solvent vehicles on the cutaneous penetration of the low molecular weight toxins, and (iv) modify the <u>in vitro</u> experimental system for skin penetration for use in studies of <u>in vitro</u> penetration across mucosal membranes. Toxins to be studied include the brevetoxins, microcystins, lyngbyatoxin A and debromoaplysiatoxin. Progress made during Year 3 of this contract is summarized below, ..			
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19. Abstract Continued

- I. The penetration and distribution of [^3H]PbTx-3 into pig skin was compared using in vivo and in vitro methods. The dose used in each topical study was 0.3 to 0.4 $\mu\text{g}/\text{cm}^2$ skin, with dimethylsulfoxide (DMSO) as the vehicle. In the in vivo study, mean cutaneous absorption after 48 hr (expressed as percentage of the dose) was 11.5% (n=3). In the in vitro study, mean cutaneous absorption after 48 hr was 1.7% (n=12), when based on accumulation of radioactivity in receptor fluid, or 10.1% when based on receptor fluid and dermis. [^3H]PbTx-3 readily penetrated through the epidermis into the dermis, reaching maximal dermal accumulation at 4 hr (9.1% in vitro and 18% in vivo). At 24 hr, the amount in the dermis decreased to 2.3% and 15% in vivo and in vitro, and at 48 hr the amount in the dermis decreased to 8.4% in vitro. The results demonstrate: (1) good correlation between in vivo and in vitro skin penetration when in vitro values are calculated by summing penetrant in dermis and receptor fluid and (2) the important role of the dermis as a reservoir for a lipophilic compound in both in vivo and in vitro percutaneous absorption studies.
- II. The effect of vehicle (methanol, DMSO, and water) on penetration and distribution of brevetoxin (PbTx-3) into excised guinea pig skin was determined with flow-through diffusion cells. The dose (0.3 $\mu\text{g}/\text{cm}^2$) was dissolved in 50 μl methanol, DMSO or water, and applied to the epidermal surface at time 0. Skin penetration was calculated by summing the amount of radioactivity recovered in the dermis and receptor fluid after various periods of incubation (0.25, 1, 2, 4, 24 and 48 hr). Percutaneous penetration (expressed as percent of dose) after 1 hr of topical exposure was 2.3%, 1.6% and 0.69% for the methanol, DMSO and water groups, respectively. Percutaneous penetration after 48 hr of topical exposure was 4.7%, 15% and 6.9% for the methanol, DMSO, and water groups, respectively. During the early time periods penetration was greatest when methanol was the vehicle, and during later time periods penetration was greatest when DMSO was the vehicle.
- III. The effect of vehicle (methanol, water, or DMSO on the penetration and distribution of microcystin in human skin was determined in vitro. Discs of excised, split thickness skin (surface area = 2.8 cm^2) were mounted on static diffusion cells. At time 0, the epidermal surfaces were each dosed with 100 μg of microcystin dissolved in 50 μl of methanol, water or DMSO. Dermal surfaces of the skin discs were bathed with receptor fluid which consisted of HEPES buffered Hank's Balanced Salt Solution. The diffusion cells were incubated at 37°C for varying periods of time (4-24 hr). Skin discs were then frozen and sectioned parallel to skin surface with a microtome. Microcystin penetration into the receptor fluid and skin sections was determined with reverse phase high performance liquid chromatography. After 24 hr of topical exposure, mean penetration into the epidermis was 0.8, 2.3 and 2.3% of the dose when methanol, water or DMSO was the vehicle; and penetration into the dermis was 12, 7.2 and 13% when methanol, water or DMSO was the vehicle. At each time interval, regardless of vehicle, less than 1.0 percent of the dose was recovered from receptor fluid.

- IV. The permeability coefficients (K_p) of buccal mucosa and skin for tritiated water (THO) were determined. K_p of human skin (0.47×10^{-3} cm/hr) correlated favorably with the previous reports. K_p of hydrated monkey skin for THO (0.77×10^{-3} cm/hr) was not significantly different ($P > 0.05$) from K_p of hydrated human skin (0.88×10^{-3} cm/hr). K_p of monkey buccal mucosa for THO (6.15×10^{-3} cm/hr) was significantly greater than that for monkey skin. Penetration and disposition of [^3H]PbTx-3 into layers of monkey buccal mucosa and skin was determined. [^3H]PbTx-3 (5-7 uCi) dissolved in 2 ml of water was applied to epithelial/epidermal surface (2.8 cm^2) at time 0. The relative percent dose recovered from the upper layers of buccal mucosa (epithelium) and skin (epidermis) varied, but at each time interval was less than 2.5 percent of the dose. At most of the time intervals (2 to 24 hr), a larger percent of the dose was recovered from the inner layer of the buccal mucosa (lamina propria) than from the inner layer of skin (dermis). After 24 hr, as much as 34 or 13 percent of the dose was recovered from lamina propria or dermis, respectively. At each time interval studied, less than 2 percent of dose of [^3H]PbTx-3 penetrated into the receptor fluid which bathed the inner surfaces of the lamina propria and dermis. The results of this study are in agreement with the generally held view that buccal mucosa is more permeable than skin.
- V. Static diffusion cells were used to study the penetration of lyngbyatoxin A through excised guinea pig abdominal skin (surface area = 2.5 cm^2). The dermis was bathed with HEPES buffered Hanks's Balanced Salt Solution with Gentamicin. The epidermis was dosed with 50 μg of lyngbyatoxin dissolved in 25 μl of DMSO. The cells were incubated in 37°C for varying time periods. High performance liquid chromatography was used to quantitate lyngbyatoxin. Recovery (expressed as percent dose) of lyngbyatoxin from the epidermis was 9.4%, 12%, 10% and 14% at 1, 4, 24 and 48 hours, respectively. Recovery from the dermis was 17%, 19%, 25% and 23% at 1, 4, 24 and 48 hours respectively. Less than 0.3% of the dose of lyngbyatoxin was found in the receptor fluid at each time period. Previous studies have found that summing the amounts of a compound found in the dermis and receptor fluid correlated well with in vivo skin absorption. These results indicate that lyngbyatoxin readily penetrated into excised skin.
- VI. An attempt was made to determine the 1-octanol/water partition coefficient (Log P) for [^3H]PbTx-3. Unfortunately, PbTx-3 was unstable in octanol but was stable in chloroform. The chloroform/water partition coefficient for [^3H]PbTx-3 was determined to be 2.20 ± 0.05 ($n=2$). A linear regression equation was used to calculate an octanol/water Log P for [^3H]PbTx-3 of 1.59. These values indicate that PbTx-3 is approximately 160 times more soluble in chloroform than water and about 32 times more soluble in 1-octanol than water.

FOREWORD

- a. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.
- b. In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication 86-23, Revised 1985).
- c. For the protection of human subjects the investigator (s) have adhered to policies of applicable Federal Law 45CFR46.

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LIST OF INVESTIGATORS

<u>Name</u>	<u>Position</u>	<u>Responsibility</u>
Barbara W. Kemppainen, PhD	Assistant Professor of Toxicology, Dept. of Physiol. and Pharmacol. Coll. Vet. Med.	Principal Investigator
Meena Mehta, MD	Research Associate, Dept. of Physiol. and Pharmacol., Coll. Vet. Med.	Skin penetration studies
Robert Stafford, BS	Graduate Student, Dept. of Physiol. and Pharmacol., Coll. Vet. Med.	Skin penetration studies
Ronald T. Riley, PhD	Research Pharmacologist, Toxicology Unit, US Dept. of Agriculture, ARS, Athens, GA	Statistical analyses
William G. Reifenrath, PhD	Research Chemist, Letterman Army Institute of Research, Presidio of San Francisco, CA.	Co-investigator for <u>in vivo</u> skin penetration studies
Hirota Fujiki	Chief, Cancer Prevention Division, National Cancer Center Research Institute, Tokyo, Japan	Supply lyngbyatoxin A for <u>in vitro</u> skin penetration studies

METHODS FOR IN VITRO SKIN ABSORPTION STUDIES OF A LIPOPHILIC
TOXIN PRODUCED BY RED TIDE

Barbara W Kemppainen^{*1}, William G Reifenrath², Robert G Stafford¹,
and Meena Mehta¹,

¹Department of Physiology and Pharmacology, College of Veterinary
Medicine, Auburn University, Auburn, AL, 36849 and

²Division of Cutaneous Hazards, Letterman Army Institute of
Research, Presidio of San Francisco, CA, 94129.

STATEMENT OF PROBLEM

The purpose of this contract is to determine the amount of risk associated with dermal exposure of humans to low molecular weight toxins. Because of the extreme toxicity of these compounds, it is not feasible to measure their cutaneous absorption in human subjects. Therefore, these studies are being performed with excised human and guinea pig skin. The compounds being studied (brevetoxin [PbTx-3], microcystin, and lyngbyatoxin A) are all lipophilic, which presents problems when using traditional in vitro methods to measure skin penetration (see BACKGROUND section for details). Consequently, it was necessary to modify the in vitro skin penetration methods to be used for this contract work. The purpose of this study was to determine if in vitro methods can be used to accurately predict in vivo skin penetration and retention of PbTx-3. The specific objective of this study was to compare in vivo skin penetration and retention by PbTx-3 with

several in vitro methods for determining skin penetration and retention by PbTx-3.

BACKGROUND

In vitro methods are widely used to measure skin absorption of xenobiotics (9). In general these in vitro methods involve mounting disks of excised skin on diffusion cells, applying the xenobiotic to the epidermal surface and quantitating accumulation of penetrant in an aqueous solution (receptor fluid) which bathes the dermal surface (10,11,12). The validity of using in vitro methods to measure percutaneous absorption has been evaluated by comparing in vivo and in vitro skin absorption of compounds of varying physical and chemical properties. The degree of correlation between in vivo and in vitro skin penetration depends on the compound and methodology (13,14). The ratio of in vivo to in vitro skin penetration increases for more lipid soluble compounds (10). It has been hypothesized that this discrepancy is due to limited solubility of the lipophilic penetrant in the hydrophilic receptor fluid (15). This hypothesis is substantiated by the observation that dermal accumulation of lipophilic penetrants is significantly greater in vitro than in vivo (16). An alternate explanation would be the poor diffusability of lipophilic or high molecular weight compounds in the dermis (17). This increased diffusional resistance in the dermis is exaggerated in the in vitro system due to the lack of functional vasculature to remove penetrant from dermal tissue. This alternate explanation is supported by reports that in vitro skin penetration by hydrophobic compounds is not altered (10) or

minimally increased (15,18) when saline receptor fluid is replaced with serum. In vitro skin penetration by the hydrophobic compound hexachlorophene was increased by a factor of two, even though the solubility of hexachlorophene in serum is 500 times greater than in water (18). Several modifications of the in vitro methodology have been used in attempts to resolve the discrepancies between in vivo and in vitro skin penetration of lipophilic compounds. One alternative method involves using surfactants or solvents as the receptor fluids (15,19). The disadvantage of using non-physiologic receptor fluid is that cutaneous metabolism is disrupted (7), which can in turn alter percutaneous penetration (11). Another alternative method is to measure dermal and receptor fluid levels of penetrant at different time intervals in order to approximate in vivo penetration (20).

RATIONALE USED IN CURRENT STUDY

In vivo and in vitro methods were used to quantify the skin penetration and retention of [^3H]PbTx-3. Dimethylsulfoxide (DMSO) was used as the vehicle for the topical studies in order to determine the amount of skin penetration during a worst case scenario, e.g., if a laboratory worker was extracting brevetoxin from cultured algae and spilled brevetoxin dissolved in a solvent on his skin. Weanling pig skin was used as a model for human skin because it has similar permeability properties (3).

EXPERIMENTAL METHODS

MATERIALS

Unlabeled PbTx-3 and (42-3H)PbTx-3 (see Figure 1 for chemical structure) were obtained from Dr. Daniel Baden, University of Miami, Miami, FL. The radiolabeled PbTx-3 had a specific activity of 12 to 14 Ci/mmol. For some of the topical studies, the specific activity of the [3H]PbTx-3 was decreased to 0.9 to 1.8 Ci/mmol by adding unlabeled PbTx-3. The radiochemical purity was determined prior to each experiment, using high performance liquid chromatography (HPLC). The HPLC methods involved using a uBondapak C18 reverse phase column (Waters, Milford, MA), mobile phase of methanol:water (85:15,v/v), flow rate of 0.5 ml/min, monitoring ultraviolet light absorbance at 215 nm and monitoring radioactivity with a FLO-ONE radioactive flow detector (Radiomatic, Tampa, FL). The retention time (Rt) of [3H]PbTx-3 was 4.0 to 4.2 min. The radiochemical purity was determined to be greater than 98%. The contaminant peak had a Rt of 1.0 to 1.3. Previous studies have shown that the [3H]PbTx-3 is stable in DMSO and methanol at 37 C for at least 48 hr (4).

Animals

Weanling pigs weighing between 9 and 17 kg were used for the studies. Female Yorkshire pigs obtained from Boswell Laboratory Animals (Corcoran, CA) were used to determine the in vivo percutaneous penetration of PbTx-3. Male and female Yorkshire-Duroc-Landrace cross-bred pigs obtained from Auburn University Swine Production Unit (Auburn, AL) were used to determine in vivo

skin retention and in vitro skin penetration and retention. The skin penetration and retention of [3H]PbTx-3 in Yorkshire and cross-bred pig skin was compared using in vitro methods. The skin barrier properties of these two skin types were found to be similar (results not shown).

In vivo percutaneous penetration of PbTx-3

This study was divided into two phases. Firstly, the disposition of radiolabel in urine, feces, liver, kidney, spleen and muscle was determined after subcutaneous administration of [3H]PbTx-3 to three pigs. Secondly, the disposition of radioactivity following topical administration of [3H]PbTx-3 to three additional pigs was determined. Percutaneous penetration (percent of applied dose) was determined by dividing the amount of radioactivity excreted in the urine and feces after topical application by the corresponding amount excreted after subcutaneous administration and multiplying the result by 100.

Radiolabeled PbTx-3 (1.1 ug, 18 uCi) dissolved in 0.5 ml propylene glycol was administered to three pigs subcutaneously. The pigs were then housed in stainless steel metabolism cages for approximately 3 days. Urine and feces were collected twice a day. The pigs were then sedated by intramuscular injection of a mixture of xylazine HCl (3 mg/kg, Rompun, Miles Laboratories, Shawnee, KS) and ketamine HCl (20 mg/kg, Vetalar, Parke-Davis, Morris Plains, NJ), and euthanized by an intravenous injection of T-61 Euthanasia solution (American Hoechst Corp, Somerville, NJ). The liver, kidney, spleen and a portion skeletal of muscle

(pectoralis) were removed from each pig. Radioactivity in urine samples was determined by mixing an aliquot (1 ml) of each urine sample with 10 ml scintillation cocktail (Optifluor, Packard Instrument Company, Downers Grove, IL). Radioactivity was measured with a Packard 1500 scintillation spectrophotometer fitted with an external standard. The feces were freeze-dried and samples placed in zip-lock polyethylene bags and ground to a fine powder with a teflon rolling pin. A 0.2 g aliquot was taken from each sample and mixed with 0.2 g of cellulose powder and oxidized in a Packard Model 306 Sample Oxidizer. Radioactivity in these vials was then determined by standard liquid scintillation counting (LSC). The liver, kidney, spleen and portion of skeletal muscle were processed as described for fecal samples, and radioactivity was determined similarly. The percentage of the dose in the total skeletal muscle mass at death was calculated based on pig body weight and published values for the body composition of pigs 28 d postnatal (5).

Pigs were prepared for the topical studies by clipping the hair (Oster Model A-2, Milwaukee, WI) on the upper back and marking a square application site (5 cm x 5 cm). The dose (0.4 ug [3H]PbTx-3/3 ul DMSO/cm², 20 uCi) was applied to the application site with a blunt tipped glass syringe (Hamilton, Reno, NV). A non-occlusive protective patch was constructed and placed over the application site as previously described (6). The patch kept the application site from contacting the metabolism cage and prevented radioactive exfoliated skin from contaminating urine and feces. The pigs were housed in metabolism cages for

approximately 4 days and urine and feces collected as described above. On day 2 the protective patches were removed and replaced with new patches. On day 3 (48 hr post application) the patches were removed and the site of application decontaminated with cotton moistened with soap and water. The radioactivity in the patches and cotton swabs was recovered by extraction with methanol and quantified by LSC. Pigs were sedated by an intramuscular injection of acepromazine maleate (0.2 mg/kg, Ayerst Laboratories, New York, NY) and euthanized by an intravenous injection of T-61. The skin from the application site was removed and sectioned into epidermis (100 um), dermis (approximately 2000 um), and subcutaneous fat with an electric dermatome (Brown Electrodermatome Model 901, Zimmer-USA, Warsaw, Ind.). As each successive layer was removed, the skin layer was placed in a scintillation vial which contained 10 ml of methanol. The dermatome cutting surface was decontaminated with methanol moistened cotton after each layer was cut. The cotton swab was then extracted with methanol and the amount of compound measured was added to that contained in the previous layer of skin. The dermis was removed with two passes of the dermatome set to cut a 1000 um layer. The remaining tissue consisted of subcutaneous fat. Skin layers were extracted by sonication in methanol for 10 min. An aliquot of the methanol was mixed with counting solution for LSC.

In vivo percutaneous retention of PbTx-3

Four pigs were prepared for this study by clipping the hair on their upper backs and marking four rectangular application sites (2 cm x 4 cm) on each pig. The dose (0.4 ug [3H]PbTx-3/3 ul DMSO/cm², 3.1 uCi) was applied to each application site at 0.25, 1.0, 2.0, 4.0, or 24 hr prior to when the pigs were euthanized. The application sites were covered with a non-occlusive protective patch (see above for description). The pigs were housed in steel metabolism cages during the topical exposure period. The pigs were sedated with an intramuscular injection of a mixture of xylazine and ketamine (see above for dose), and euthanized by an intravenous injection of T-61. The protective patches were removed and skin from the upper back excised and cut into sections (6 cm x 12 cm), each of which contained one of the application sites. The surface of each application site was decontaminated with cotton moistened with soap and water. The skin was sectioned into epidermis (100 um), dermis (approximated 2000 um) and subcutaneous fat. The skin sections and cotton swabs were each extracted with methanol and an aliquot of each extract was counted with LSC.

In vitro skin penetration and retention of PbTx-3: Determined with skin disks incubated in diffusion cells

Skin was excised from the upper back of female and male weanling pigs. Split-thickness skin consisting of the epidermis and a portion of the dermis (total thickness of 900 um) was prepared with a Padgett Electro Dermatome (Padgett Instruments,

Kansas City, MO) and mounted on glass flow-through diffusion cells (Laboratory Glass Apparatus, Berkeley, CA). The diffusion cells have a water jacket which maintains the receptor fluid at 37°C. Each cell exposes 1 cm² of skin surface area and has a receptor fluid volume of 3 ml. Hepes-buffered Hanks's balanced salt solution (HHBSS, GIBCO, Grand Island, NY) was used as the receptor fluid which bathes the dermal surface of the skin disk. HHBSS has been shown to maintain the viability (as measured by glucose utilization) of excised skin in diffusion cells (7). The receptor fluid was pumped through the diffusion cells at the rate of approximately 5 ml per hour. The skin surfaces were each dosed with 0.3 ug [3H]PbTx-3/6 ul DMSO/cm² (5 uCi), using a Hamilton microliter syringe with a blunt tip. The epidermal surfaces were exposed to ambient air (22°C) during the entire length of the experiment. Penetration of [3H]PbTx-3 into skin layers and receptor fluid was determined by incubating the diffusion cells for various time intervals (0.25, 1.0, 2.0, 4.0, 24, and 48 hr) and measuring radioactivity in skin layers and receptor fluid. Accumulated radioactivity (PbTx-3 and metabolites) in receptor fluid was determined by collecting receptor fluid at hourly intervals and analyzing radioactivity in each sample with standard LSC techniques. The epidermal surfaces were decontaminated with cotton moistened with soap and water to determine the amount of toxin remaining on the skin surface. The area of the skin dosed with toxin was cut away from the perimeter portion of the skin. The perimeter portion of the skin was extracted with methanol to determine if part of the

applied dose had diffused to the outer portion of the skin disk and was not available for diffusion through the skin into the receptor fluid. The central portion of each skin disc was mounted on embedding blocks (Thomas Scientific, Swedesboro, NJ) with Tissue-Tek OCT Compound (American Scientific Products, Stone Mountain, GA), frozen, and sectioned parallel to the skin surface with a microtome (Bausch and Lomb, Optical Co, Rochester, NY). The epidermis (100 μ m) and dermis (remaining 800 μ m) were each extracted twice with methanol. The radioactivity in receptor fluid, extracts of skin wash and skin sections was summed to determine total recovery of the applied dose.

In vitro skin penetration and retention of PbTx-3: Determined with skin slabs incubated on absorbent material moistened with tissue culture media

Full-thickness skin (including subcutaneous fat) was obtained from the upper back of two female weanling Yorkshire pigs. Rectangular pieces (6 cm x 12 cm) were placed dermal side down on stainless steel trays lined with gauze pads moistened with RPMI media (GIBCO). The 2 cm x 4 cm areas for toxin application were outlined with an alcohol marker. The toxin (0.4 μ g [3 H]PbTx-3/3 μ l DMSO/cm², 2.3 uCi) was applied to each application site. The dosed skin segments were divided into 4 groups which were incubated at room temperature (22°C) for different periods of time (0.25, 1.0, 2.0 and 4.0 hr). The distribution of radiolabeled toxin into layers of the skin was determined by

first decontaminating the surface of the skin with cotton moistened with soap and water to recover $[3H]PbTx-3$ which remained on the skin surface. The skin was layered into epidermis, dermis, and subcutaneous sections and extracted with methanol as described for in vivo skin retention studies. A portion of the methanol extracts were mixed with counting solution for LSC. The remaining portions of the methanol extracts were individually evaporated to dryness under a steady stream of nitrogen and immediately dissolved in 100 μ l methanol. The radiochemical composition of the skin extracts was determined with HPLC (see first paragraph of MATERIALS AND METHODS section for HPLC method).

A control group was included in order to determine if radioactivity on the dermatome cutting surface was contaminating subsequent skin sections and thus confounding the results. This was done by placing skin segments dermal side down on absorbent material moistened with RPMI medium (as described above). The epidermal surfaces of skin in the treated group were dosed with 0.4 μ g $[3H]PbTx-3/3$ μ l DMSO/ cm^2 and the skin segments in the other group were dosed only with the vehicle (DMSO). All of the skin segments were incubated for 45 min and the skin surfaces were decontaminated as described above. The dermatome was used to: (i) remove the epidermis of one of the skin segments which was dosed with DMSO only and (ii) remove the epidermis of one of the skin segments which had been dosed with $[3H]PbTx-3$. The dermatome cutting surface was decontaminated with cotton moistened with methanol and was used to remove a section of the

dermis from the skin segment which had been dosed with DMSO only. This procedure was used to determine the amount of toxin which was carried over from slicing the epidermal layer (which had the highest level of radioactivity) to the dermal layer.

RESULTS

In vivo percutaneous penetration of PbTx-3

The disposition of radioactivity following subcutaneous and topical application of [3H]PbTx-3 to weanling pigs is shown in Table 1. The mean percent dose cutaneously absorbed (11.5 ± 1.7 [SE]) during 48 hr of topical exposure was calculated by dividing the percent dose excreted following topical administration (2.3 ± 0.3) by the percent dose excreted following subcutaneous administration (20 ± 6.9), and multiplying by 100. Analysis of radioactivity in selected organs following subcutaneous administration indicated that muscle had the largest concentration, followed by liver, spleen and kidney. The total recovery of the dose was low (36% and 80%, following subcutaneous and topical administration, respectively). The low recovery rates were probably due to residual radioactivity in carcass components which were not analyzed.

In vivo percutaneous retention of PbTx-3

The disposition of radioactivity into skin layers following topical application of [3H]PbTx-3 to weanling pigs is shown in Figure 2. Nine percent of the dose had penetrated into the

dermis during the first four hours of exposure. Therefore, 78% of the dose cutaneously absorbed during 48 hr had penetrated into the dermis within the first 4 hr of exposure. At each time interval examined the amount of radioactivity in the epidermis was greater than the amount in the dermis. The total recoveries of the applied doses were 61 to 94 percent. The radioactivity in the protective patches was not determined and this may be responsible for the low total recoveries.

In vitro skin penetration and retention of PbTx-3: Determined with skin disks incubated in diffusion cells

The disposition of radioactivity into skin layers and receptor fluid following topical application of [3H]PbTx-3 to excised skin mounted on flow-through diffusion cells is shown in Figure 3. In contrast to in vivo disposition into skin layers, the amount of radioactivity in the dermis is greater than the amount in the epidermis at all time intervals which were studied except 48 hr. The mean percent dose which penetrated into the dermis and receptor fluid was 8.4 ± 0.81 (SE) and 1.7 ± 0.50 (respectively) after 48 hr of exposure. Recovery of radioactivity in the receptor fluid is indicated only at times 1.0, 24 and 48 hr, because it was not possible to indicate on this figure the very small levels of radioactivity (0 to 0.005 percent of the dose) in the receptor fluid at the other time intervals.

In vitro skin penetration and retention of PbTx-3: Determined with skin slabs incubated on absorbent material moistened with tissue culture media

The disposition of radioactivity into skin layers during topical exposure of excised skin slabs to [3H]PbTx-3 is shown in Figure 4. The accumulation of radioactivity in the epidermis and dermis was considerably less than that observed in the in vivo or in vitro diffusion cell experiments. Accumulation of radioactivity in the epidermis was greater than that in the dermis, which is consistent with the in vivo finding. However, the amount (percent of the dose) in the epidermis (3.5 to 7.2) and dermis (1.6 to 2.9) in the skin slab experiment was approximately half the amount in the epidermis (8.0 to 27) and dermis (2.7 to 9.1) in the in vivo study.

Results from the control study indicate that radioactivity remaining on the dermatome cutting surface after decontamination with methanol resulted in contamination of the dermis with 0.13 percent of the dose. In other words, less than 1/10th of the 2 to 3 percent of the dose extracted from the dermis was due to contamination of the dermatome cutting surface.

Analysis of the radiochemical composition of extracts of the skin washes and skin sections indicated that greater than 95% of the radioactivity in each sample was associated with PbTx-3.

DISCUSSION

The percutaneous penetration of [3H]PbTx-3 during 48 hr of exposure has been compared using in vivo and in vitro (diffusion cells) methods. PbTx-3 is a lipophilic, large molecular weight compound. The solubility of PbTx-3 in water is approximately 100 mg/l (Baden, personal communication). The molecular weight of PbTx-3 is 896 (8). In vivo and in vitro percutaneous penetration of PbTx-3 (11.5 and 10.1 percent of the dose, respectively) correlated well when the in vitro value was calculated by summing radioactivity in the dermis (8.4%) and receptor fluid (1.7%). However, in vitro skin penetration of PbTx-3 underestimated in vivo penetration by a factor of 7 when the in vitro value was based only on accumulation of radioactivity in receptor fluid.

The percutaneous retention of [3H]PbTx-3 during initial exposure periods was compared using an in vivo method and two in vitro methods. In the in vivo and in vitro (diffusion cell) experiments the accumulation of [3H]PbTx-3 in the dermis reached a maximum level after 4 hr of exposure (9.1 and 18 percent of the dose, respectively). Formation of a dermal reservoir is contrary to the generally held belief that during percutaneous penetration most of the penetrant is absorbed by the capillary bed at the dermo-epidermal junction (15, 19, 21, 22). The existence of a dermal reservoir in vivo and in vitro demonstrates the important role the dermis plays in percutaneous absorption. After 24 hr of exposure, the in vivo dermal reservoir was reduced to 2.3 percent of the dose. This was probably due to removal of

[3H]PbTx-3 from the dermis via dermal vasculature; and reduced penetration of [3H]PbTx-3 from epidermis into dermis due to diminished vehicle effects. The enhancement of percutaneous penetration by DMSO is temporary and reaches complete extinction three hr after treatment due to absorption of DMSO by circulation (23). Between 4 and 48 hr, the sum of the radioactivity in the dermis and receptor fluid of the in vitro diffusion cells had decreased from 18 to 10 percent of the dose. During this same time period the radioactivity in the epidermis increased from 4.8 to 9.7 percent of the dose. This sequence of events suggests there is a "back diffusion" or exsorption of [3H]PbTx-3. In vivo percutaneous exsorption of xenobiotics during topical exposure to DMSO has been reported (24). The larger dermal reservoir observed in vitro than in vivo is consistent with previous reports (16, 25) and is thought to be due to the absence of functional vasculature in vitro and limited solubility of lipophilic penetrants in aqueous receptor fluid. The penetration of [3H]PbTx-3 into epidermis, dermis and subcutaneous fat was considerably less for the in vitro skin slab method than in vivo or in vitro diffusion cell methods. This difference in percutaneous penetration is probably due to the temperature differences in the skin slab (room temperature, 20° C), in vivo (37°C) and in vitro diffusion cell (37°C) methods. It is well documented that higher temperatures results in faster rates of percutaneous penetration (9).

The in vivo and in vitro skin penetration and retention of [3H]PbTx-3 in pigs is expressed as percent dose of [3H]PbTx-3

equivalents which accumulated in each sample. These calculations were based on the amount of radioactivity in the samples and do not take into account that a fraction of the radioactivity may have been associated with metabolites or breakdown product of [3H]PbTx-3. In the in vitro skin penetration and retention study greater than 95% of the radioactivity in each skin extract was associated with PbTx-3. These findings are consistent with the hypothesis that excised pig skin does not metabolize PbTx-3. These results are in agreement with the results of previous studies which indicate [3H]PbTx-3 is not metabolized by excised human and guinea pig skin (4).

CONCLUSIONS

In vitro percutaneous penetration of [3H]PbTx-3 provided an accurate indication of in vivo penetration when in vitro values were calculated by measuring dermal and receptor fluid levels of [3H]PbTx-3 in the diffusion cells. The formation of a dermal reservoir of [3H]PbTx-3 in the in vivo and in vitro studies demonstrates the important role the dermis plays in the process of percutaneous absorption.

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Table 1

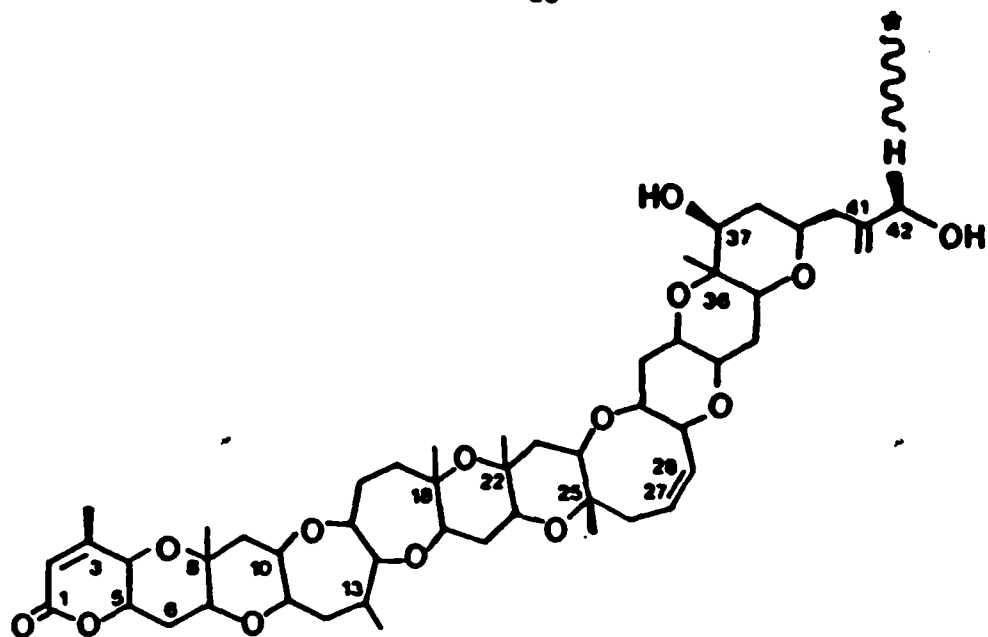
RECOVERY OF RADIOACTIVITY FOLLOWING SUBCUTANEOUS INJECTION AND
TOPICAL APPLICATION OF [3H]PbTx-3 TO WEANLING PIGS

Organ/Tissue	<u>Route of administration</u>	
	Subcutaneous	Topical
Urine	11±1.8 ^a	1.1±0.1
Feces	8.9±7.4	1.2±0.3
Muscle	12±1.5	---
Liver	3.6±0.2	---
Kidney	0.07±0.06	---
Spleen	0.12±0.01	---
Skin surface ^c	---b	34±4.8
Epidermis ^c	---	1.7±0.3
Dermis ^c	---	1.8±0.8
Subcutaneous fat ^c	---	0
Protective patch	---	39±20
Total recovery of dose	36±21	80±16

^aFigures are expressed as percentage of dose (mean ± standard error, sample size = 3).

^b---Indicates value not determined.

^cAt site of topical application.



Brevetoxin

[42-³H]PbTx-3

Figure 1. Structure of tritium labeled PbTx-3.

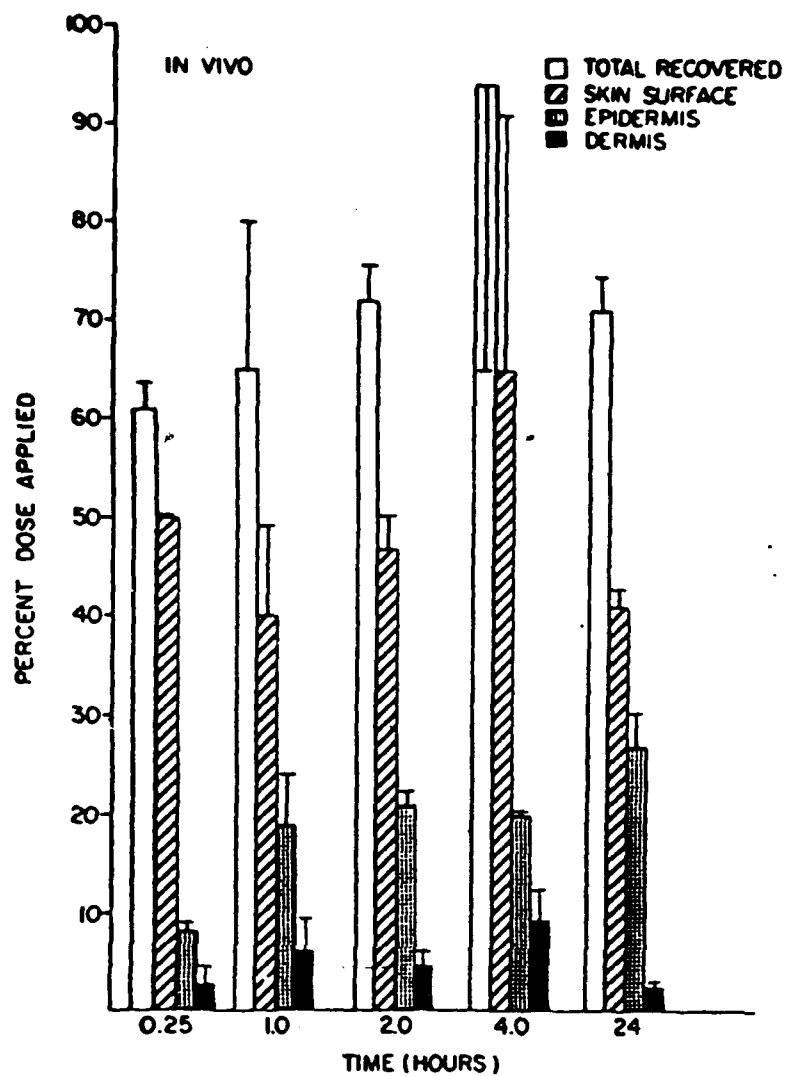


Figure 2. In vivo skin retention of PbTx-3 equivalents (percent of applied dose) following topical application to pigs.

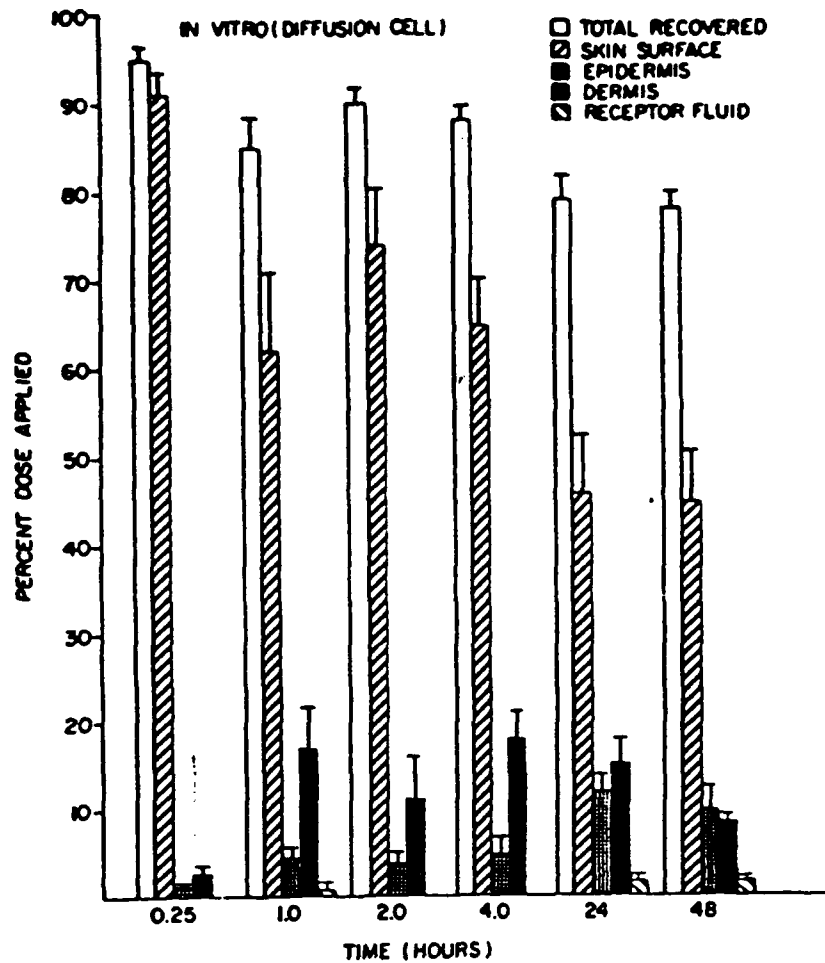


Figure 3. In vitro skin penetration and retention of PbTx-3 equivalents (percent of applied dose) following topical application to pig skin mounted on diffusion cells.

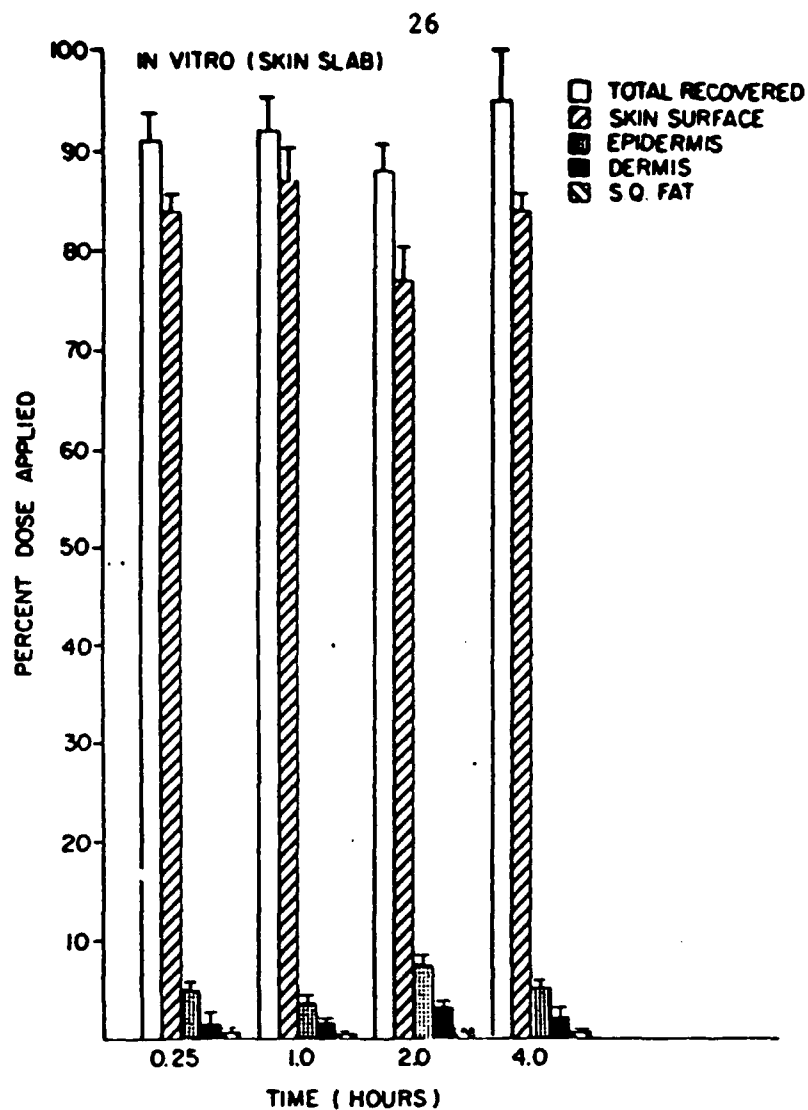


Figure 4. In vitro skin penetration and retention of PbTx-3 equivalents (percent of applied dose) following topical application to pig skin slabs incubated on absorbent material moistened with tissue culture media.

EFFECT OF VEHICLE (METHANOL, DIMETHYLSULPHOXIDE [DMSO] AND WATER) ON THE
PENETRATION AND DISTRIBUTION OF BREVETOXIN (PbTx-3) INTO GUINEA PIG SKIN IN
VITRO.

STATEMENT OF THE PROBLEM

In vitro methods have been widely used to predict in vivo penetration of compounds through skin. Many of these studies did not take into account the accumulation of penetrants in the layers of the skin (i.e., stratum corneum, epidermis and dermis), but instead skin penetration calculations were based only on the amount of penetrant recovered in the receptor fluid (1, 2). Recent studies indicate that in vitro skin penetration correlates better with in vivo skin penetration when in vitro values are calculated by summing penetrant recovered from the dermis and receptor fluid (3, 4). Therefore, the specific objective of this experiment was to use a flowing diffusion cell method to compare the penetration and distribution of brevetoxin (PbTx-3) into receptor fluid and the layers (stratum corneum, epidermis and dermis) of excised guinea pig skin. Methanol, dimethylsulfoxide (DMSO) and water were used as the vehicles.

BACKGROUND

In preliminary in vitro experiments the penetration of PbTx-3 through skin was determined by measuring accumulation of penetrant in receptor fluid (5). Calculations of in vitro skin penetration based only on the amount of the applied dose recovered from the receptor fluid significantly under estimated skin penetration by lipophilic compounds (3, 4). PbTx-3 is a lipophilic compound which has a solubility in water of approximately 100 mg/l (Baden, personal communication). Therefore it was important to determine the effect of vehicle on the penetration and distribution of brevetoxin into the layers of the skin and receptor fluid.

RATIONALE USED IN CURRENT STUDY

An in vitro method has been developed using glass flow-through diffusion cells for measuring the penetration of compounds through excised skin. The validity of using excised skin to estimate *in vivo* skin penetration by xenobiotics was tested by comparing the in vitro and in vivo skin penetration of several compounds (1, 6). It was concluded that there was good correlation between in vitro and in vivo skin penetration for most cases studied. However, there were some exceptions. The decreased correlation was attributed to low solubility of lipophilic compounds in the aqueous receptor fluid. Better agreement between in vitro and in vivo penetration through pig skin was obtained by summing the penetrant recovered from both the dermis and the penetration cell fluid to provide an adjusted *in vitro* penetration value (3). Therefore, the effect of vehicle on percutaneous penetration of PbTx-3 was determined by measuring accumulation of penetrant in the dermis and receptor fluid.

MATERIALS AND METHODS

Guinea pigs used in the study were obtained from the Auburn University laboratory animal facility. Male guinea pigs were chosen, ranging from 709-1,081 grams dead weight. The labelled compound ($[^3\text{H}]\text{PbTx-3}$) had a specific activity of 9.93 and 13.23 Ci/m mole. The radiochemical purity was 97% or greater, as determined by high performance liquid chromatography. Prior to each experiment a guinea pig was killed with CO_2 , the dead weight recorded, the hairs on the abdominal area were carefully clipped with an electric clipper and the skin removed with the aid of a scalpel blade and handle. Remaining fat or muscle was removed by blunt dissection. Disks of skin (surface area = 1 cm^2) were prepared from the skin sample with a cork borer and scissors. The skin disks were mounted on flow-through diffusion

cells designed for the study of percutaneous penetration (Laboratory Glass Apparatus, Berkely, CA). The epidermal surfaces were exposed to room air and the dermal surfaces were bathed by flowing receptor fluid. The receptor fluid (Hanks's Balanced Salt Solution [HBSS] with gentamicin) was pumped through the cells at a rate of 2.9 - 3.2 ml/hour by a peristaltic pump from a reservoir. The reservoir was continuously supplied with a slow stream of gaseous mixture of 95% O₂: 5% CO₂. A fraction collector was used to collect hourly samples of the receptor fluid, which were then transferred to glass scintillation vials for radioactive determination. When a steady rate of receptor fluid flow was reached, the epidermal surfaces were dosed with 320 ng/cm² of [³H]PbTx-3 dissolved in 50 µl of the appropriate vehicle (methanol, DMSO or water). The diffusion cells were incubated for various time periods (0.25 to 48 hr), after which the cells were disassembled. The receptor fluid from each cell was individually transferred to glass scintillation vials for determination of radioactivity by liquid scintillation (LSC) counting. At the end of each specified time period the epidermal surfaces were washed first with cotton swabs moistened with a dilute soap solution (1:100) and then with distilled water. The cotton swabs were extracted with 10 ml of methanol. The skin disks were removed, mounted on plastic embedding blocks, and stored in the freezer (-20°C) until the following day when they were sectioned into the skin layers [i.e., stratum corneum (0-15 microns), epidermis (15-100 microns), dermis (remaining portion)] with a microtome (Bau^Sch and Lomb Optical Co., Rochester, NY). Each skin layer was individually extracted with 2 ml of methanol. Duplicate samples (50 µl) of each extract of the surface washes and skin layers were placed in glass scintillation vials and prepared for radioactive counting by addition 10 ml of cocktail. The amount of radioactivity in the receptor fluid, surface washes and skin layers were

determined with a Packard liquid scintillation counter (Model 1500, Downers Grove IL). Recovery of the applied dose (expressed as percent) was calculated by summing radioactivity in the surface wash, skin layers and receptor fluid.

RESULTS AND DISCUSSION

Recovery from the Surface Wash

The results for each time period (0.25 to 48 hrs) and each vehicle (methanol, dimethylsulfoxide and water) are presented in Table 1. The mean values \pm standard errors are shown ($n = 3$).

Recovery from the skin surface was relatively constant during the first 2 hrs, but there was a consistent decline during the subsequent time periods. This was more obvious for water and DMSO than for methanol, and corresponded with increasing amounts of the applied dose penetrating into the skin layers. The penetration of PbTx-3 into and through the skin is related to the volatility and nature of the vehicles. Scheuplein et al (7) reported that the stratum corneum is most effective as a barrier when dry, less effective when hydrated, and still less effective when solvated with DMSO. Methanol rapidly evaporates from the skin surface due to its high volatility, and therefore appears to have minimal effects on penetration enhancement.

Recovery From the Skin Layers

Recovery of radioactivity from epidermal and dermal layers was the highest when DMSO was the vehicle, followed by water, and least for methanol. This finding is in agreement with Franz, (8) who found that DMSO enhanced the penetration of hydrocortisone and other compounds into the skin. The amount recovered in the skin layers increased with time, and this was most noticeable after 4 hrs.

Recovery of PbTx-3 from the stratum corneum during 4 to 48 hr time periods was greatest when water was the vehicle, followed by DMSO and least for methanol. However, in the earlier time periods (0.25 to 2 hrs) methanol and DMSO showed higher values than water for amounts penetrating into the stratum corneum. This observation may be due to too short a time period (0.25 to 2 hr) for water to adequately hydrate the stratum corneum. During longer periods of exposure (4 to 48 hr) there was enough time for greater hydration. It is known that partial hydration of the stratum corneum increased permeability in vitro (6). Therefore, more complete hydration would be expected to have a more profound effect, as supported by our observations.

In general, the percentage of the dose entering the epidermis was less than the values for the dermis and the stratum corneum for all three vehicles.

Recovery from the Receptor Fluid

Recovery from the receptor fluid was minimal for both methanol and water for all time periods. However, when DMSO was the vehicle recovery remained fairly low for the first 4 hr but then increased to mean values of $10 \pm 4.8\%$ and $3.2 \pm 1.6\%$ after 24 and 48 hr (respectively). This finding may be related to the solubilizing property of DMSO.

Total Recovery from the Applied Dose

The total amount of dose recovered at the end of each experiment ranged from 72 to 94% of the dose. The recovery was fairly consistent throughout all the time periods for both methanol and water. When DMSO was the vehicle the values were somewhat lower for the 24 and 48 hr time periods. These time periods also corresponded to low percentage recovery from the surface wash and much higher percentages penetrating into the skin layers and receptor fluid. The reasons for these lower recovery values are unknown but may be due to less efficient extraction of [^3H]PbTx-3 from skin layers than from skin surface.

CONCLUSION

The total amount of the dose which penetrated into the three layers of the skin and receptor fluid at 24 hrs when DMSO was the vehicle (46%) was more than eight times that which occurred when methanol was the vehicle (5.6%). The percentage of the dose recovered from the receptor fluid was minimal for the vehicles methanol and water and this may be due to limited solubility of the lipophilic PbTx-3 in the aqueous receptor fluid. These results indicate that PbTx-3 readily penetrates into the dermis and receptor fluid in the presence of DMSO.

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Table 1

Percentage of the Applied Dose ($[^3\text{H}]\text{PbTx-3}$) Recovered at Each Time Interval (Mean Values \pm S.E.)

LOCATION

(With Methanol as Vehicle)

	15 Mins	1 Hour	2 Hours	4 Hours	24 Hours	48 Hours
Surface Wash	87 \pm 2.5	87 \pm 1.8	88 \pm 1.0	86 \pm 0.84	87 \pm 3.1	83 \pm 2.7
Stratum Corneum	1.6 \pm 1.1	1.9 \pm 0.57	2.7 \pm 0.79	1.6 \pm 0.50	2.3 \pm 0.074	4.0 \pm 1.6
Epidermis	0.94 \pm 0.74	2.0 \pm 0.62	1.5 \pm 0.15	1.6 \pm 0.24	1.0 \pm 0.26	1.4 \pm 0.25
Dermis	0.68 \pm 0.54	2.3 \pm 0.67	0.86 \pm 0.16	3.4 \pm 1.9	2.0 \pm 0.49	4.3 \pm 0.73
Receptor Fluid	0.00050 \pm 0.00012	0.010 \pm 0.0038	0.0049 \pm 0.0015	0.052 \pm 0.021	0.32 \pm 0.13	0.41 \pm 0.063
Total Recovered	91 \pm 0.94	93 \pm 0.58	94 \pm 1.81	93 \pm 2.5	90 \pm 3.81	89 \pm 3.4

(With DMSO as Vehicle)

	15 Mins	1 Hour	2 Hours	4 Hours	24 Hours	48 Hours
Surface Wash	77 \pm 7.3	79 \pm 0.29	76 \pm 1.6	72 \pm 4.0	40 \pm 4.0	51 \pm 5.5
Stratum Corneum	0.61 \pm 0.14	1.8 \pm 0.87	2.7 \pm 0.95	2.5 \pm 0.92	5.2 \pm 0.94	3.5 \pm 1.1
Epidermis	0.55 \pm 0.12	0.99 \pm 0.46	2.0 \pm 0.51	3.9 \pm 1.2	4.8 \pm 1.8	2.0 \pm 0.47
Dermis	0.37 \pm 0.036	1.6 \pm 0.20	7.9 \pm 4.9	6.0 \pm 0.61	16 \pm 2.6	12 \pm 4.9
Receptor Fluid	0.0014 \pm 0.00048	0.031 \pm 0.019	0.040 \pm 0.013	0.14 \pm 0.090	10 \pm 4.8	3.2 \pm 1.6
Total Recovered	78 \pm 7.1	83 \pm 1.6	88 \pm 3.4	84 \pm 3.0	77 \pm 2.3	72 \pm 6.8

(With water as Vehicle)

	15 Mins	1 Hour	2 Hours	4 Hours	24 Hours	48 Hours
Surface Wash	73 \pm 5.1	80 \pm 0.46	81 \pm 2.4	65 \pm 2.9	67 \pm 2.8	69 \pm 2.7
Stratum Corneum	1.5 \pm 0.58	1.4 \pm 0.58	1.7 \pm 0.58	7.7 \pm 3.0	6.3 \pm 1.2	5.1 \pm 1.9
Epidermis	0.57 \pm 0.27	0.81 \pm 0.24	1.1 \pm 0.38	4.2 \pm 1.2	3.0 \pm 0.51	2.6 \pm 0.82
Dermis	0.18 \pm 0.068	0.69 \pm 0.28	0.61 \pm 0.16	3.6 \pm 0.59	5.1 \pm 1.3	5.3 \pm 0.48
Receptor Fluid	0.0022 \pm 0.00048	0.0039 \pm 0.0017	0.010 \pm 0.0069	0.050 \pm 0.018	1.1 \pm 0.39	1.6 \pm 0.87
Total Recovered	75 \pm 5.3	83 \pm 1.3	85 \pm 2.9	81 \pm 2.5	82 \pm 2.1	83 \pm 2.1

**EFFECT OF VEHICLE (METHANOL, DIMETHYLSULFOXIDE AND WATER) ON
PENETRATION AND DISTRIBUTION OF MICROCYSTIN THROUGH EXCISED HUMAN
SKIN**

M. Mehta, B.W. Kemppainen and R.G. Stafford

**Department of Physiology and Pharmacology, College of Veter-
inary Medicine, Auburn University, AL 36849**

STATEMENT OF PROBLEM

The primary purpose of this study was to determine the effect of vehicle [methanol (MEOH), dimethylsulfoxide (DMSO) and water] on penetration and distribution of microcystin through excised human skin. A portion of this work was done last year; however additional studies were needed because i) penetration of microcystin was previously determined only for a single time interval (e.g., 48 hr), ii) skin discs previously were not sectioned to determine the distribution into various layers. Therefore, further work was done this year to determine these additional parameters.

BACKGROUND

Microcystin is a blue green algae (cyanobacteria) toxin

produced by some strains of Microcystis aeruginosa [1,2]. Several types of microcystins have been isolated: microcystin-LR, -YR and -RR, (also called Cyanoginosins-LR, -YR and -RR [3,4]. Microcystin-LR is the principal toxic peptide produced by laboratory isolate of M. aeruginosa. This toxin is a cyclic heptapeptide (Fig.1) containing several unusual amino acids and has a molecular weight of 994 daltons [3,5]. Toxic blooms of several strains of blue green algae are found worldwide in both natural and man made fresh water lakes [6]. The most favorable conditions for a bloom to occur are warm, dry, low wind days of summer and early fall. Increased pollution in urban, recreational and agricultural water sources seems to contribute to the growth of toxic and nontoxic blooms [7]. Consumption of toxins in water and bloom mass have been implicated in the loss of live stock and wild animals in several countries throughout the world [1,7,8], as well as human intoxication [9,10]. Rats and mice injected with acutely toxic doses of M. aeruginosa cells or toxin extract die within 1 to 3 hr [11]. Liver damage has been noted as early as 15 minutes and it has been suggested that liver damage is a direct effect of microcystin on the hepatocyte membrane [12]. The immediate cause of mortality in acutely dosed animals is hemorrhagic shock [13,14].

It has been reported in various studies that microcystin poses a health risk to humans [10]. The incidence of fatal human poisonings by blue green algal toxins is not known. There is evidence that contact irritations and gastroenteritis occur when

swimmers come into contact with toxic blue green algae cells or water containing the released toxin [7]. It is not known if the toxin(s) were absorbed percutaneously or via ingestion. There is increasing concern that toxic blooms might pose a health risk to people through 1) drinking contaminated water and 2) recreational water. So far, no study has been reported to determine if skin exposure to water contaminated with microcystin would result in the penetration of microcystin through human skin. Due to extreme toxicity, it is not possible to conduct in vivo studies in humans. The purpose of this study was to determine if microcystin can penetrate excised human skin. The degree of correlation between in vivo and in vitro cutaneous absorption of compounds depends on the compounds, species and methodology used in investigations [15,16,17]. In many cases there is a good correlation between in vivo and in vitro penetration. The purpose of the present study was to determine the extent to which microcystin penetrates excised human skin. The specific objectives of this study were to determine: i) the effect of vehicle [MEOH, DMSO and water] on penetration of microcystin through human skin and ii) distribution of microcystin into various layers of human skin, e.g. stratum corneum, epidermis and dermis.

MATERIALS AND METHODS

Microcystin-LR was generously supplied by Dr. J.G.Pace, U.S. Army, Frederick, MD. Microcystin supplied was further purified by

high pressure liquid chromatography (HPLC) to a purity of greater than 99 %. The HPLC method followed was a modification of the previously described methods [5,15]. Reversed phase chromatography was performed with a unit from Waters Associates (Milford, MA). Separation was performed on either a Waters uBondapak C18 steel column (10 μ m, 2 mm x 30 cm) or a Hypersil ODS column (5 μ m, 50 mm x 4.6 mm, Keystone Scientific, Inc., State College, PA). The eluant [0.1 M ammonium acetate (pH 4 to 6): acetonitrile (75:25, v/v)] and was pumped at a flow rate of 0.5 ml/min. Ultraviolet (UV) absorbance was monitored at 240 nm. The microcystin peak had a retention time of 11 ± 1 min (mean \pm SD, Fig.2).

Six ICR female mice weighing 27 ± 0.5 g (mean \pm SD) were used to test the toxicity and verify the identity of the microcystin peak. After injection of the microcystin into the HPLC, eluant was collected from 10 to 12 min with a fraction collector (ISCO Inc., Lincoln, Nebraska). The collected fraction was blown to dryness under a gentle stream of nitrogen. The residue was dissolved in normal saline. ICR mice were dosed intraperitoneally (i.p.) with 20, 40 and 100 μ g/kg of microcystin in 20, 40 and 100 μ l of normal saline, respectively. For the control group, normal saline was injected into the HPLC and the eluant was collected at the same retention time as that of the microcystin peak (10 to 12 min). The collected fraction was evaporated to dryness under a nitrogen stream, resuspended in normal saline and injected into the control mice (n = 3). This

was done to verify the toxicity of the HPLC eluant was not due to an HPLC contaminant, but was due to the microcystin eluting at 10 to 12 min. No effect was observed in the control group. The LD50 was determined to be 40 ug/kg, i.p., and is consistent with the reported value [16].

The stability of microcystin in the receptor fluid was evaluated by mounting Teflon discs instead of skin, on two diffusion cells in each treatment group. The receptor fluid was dosed with microcystin (5 ug/100 ul of water, 100 ug/25ul of DMSO, 100ug/50 ul of MEOH. The diffusion cells with Teflon discs were incubated along with ^{DIFFUSION} cells with skin discs, in an environmental chamber (37°C). Each treatment group was replicated at least twice. Samples from receptor fluid were prepared and analyzed in the same manner as the receptor fluid samples collected from the diffusion cells with skin discs.

Human abdominal skin was obtained from autopsies (performed within 24 hr of death) at local hospitals. The skin from 5 males (ages 47 to 79 years) was stored in airtight plastic bags at 5 °C for up to 5 days prior to an experiment. Previous studies have shown that the permeability of skin did not change significantly when stored in this manner for up to 10 days [17]. Loose subcutaneous fat was removed from excised skin. Split-thickness human skin (thickness 800 um) was prepared with a dermatome [Padgett Dermatome, Division of Kansas City Assemblage Co, Kansas, MO].

Discs of excised skin, each 2.8 cm² in diameter, were

mounted on static teflon diffusion cells [18] consisting of an upper and lower chamber. The lower chamber (dermal side) had a volume of 2.6 ml and was filled with receptor fluid. The receptor fluid was Hanks's Balanced Salt Solution (HBSS) with HEPES buffer and gentamicin (50 mg/lit). HBSS was bubbled with 95% oxygen and 5% carbon dioxide to maintain the viability of skin discs [19]. The skin discs were placed horizontally between the two chambers so that the dermal side was bathed by the receptor fluid and the epidermal surface was exposed to ambient conditions in an environmental chamber. Temperature and relative humidity during the length of experiment was $36 \pm 3^{\circ}\text{C}$ and $29 \pm 5\%$ (mean \pm SD), respectively.

In all the experiments skin from each specimen was mounted on at least two diffusion cells in each group (3 to 4 cells/group) so that the variations in skin penetration were evenly distributed. Each experiment was repeated three times. At time 0, the epidermal surfaces of human skin were dosed with 34 - 36 $\mu\text{g}/\text{cm}^2$ of microcystin dissolved in 50 μl of ME OH, DMSO or water. The epidermal surfaces were exposed to ambient conditions in the environmental chamber.

In order to determine if constituents leaching out of skin would interfere with the HPLC determination of microcystin, a control group of skin discs was dosed only with vehicle (i.e., with 50 μl of ME OH, DMSO or water). Control diffusion cells containing the skin discs dosed with only vehicle were incubated (at 37°C) along with cells containing skin discs dosed with

microcystin dissolved in different vehicles. At the end of the experiments, receptor fluid and skin discs dosed with vehicle only were analyzed in a similar manner as the skin discs dosed with microcystin dissolved in these different vehicles.

At the end of each exposure period (4, 12 and 24 hr), the epidermal surfaces were washed with soap and water (1:10 dilution) to determine the amount of the dose remaining on the skin surface. Skin discs were immediately frozen (-20° C) at the end of exposure period and sectioned with a microtome (Bauch & Lomb, Optical Co., Rochester N.Y.) at a setting of 25 μ m. The skin discs were extracted twice with 5 ml acetonitrile (by sonication), centrifuged, the supernatant was then collected and dried under a gentle stream of nitrogen. The residues were immediately dissolved in 200 μ l methanol. Prior to the injections into the HPLC, the receptor fluids from each experiment were washed on 3 ml C18 solid phase extraction columns (SPE, J.T. Baker, Phillipsburg, NJ). Recovery of microcystin from SPE columns was determined to be 95 ± 3 percent (mean \pm SD). Each column was primed with 3 ml of methanol followed by 6 ml HBSS. Receptor fluid was then aspirated and microcystin eluted with 2 ml of methanol. The eluant was evaporated to dryness under nitrogen stream and resuspended in 200 μ l methanol for injection into HPLC.

Total recovery of the dose applied to each skin disc was calculated by summing the amount recovered from skin extracts, amount penetrated into receptor fluid and amount recovered from

skin wash.

RESULTS AND DISCUSSION

The effect of vehicle (MEOH, DMSO and water) on penetration and distribution of microcystin into excised human skin is listed in Table 1. At each time period studied, e.g., 4, 12 and 24 hr, the largest portion of the dose was left on the epidermal surface. For each vehicle and exposure period studied, the accumulation of microcystin in the receptor fluid was less than 1.5 % of the applied dose. At the 4 and 12 hr time period, the greatest penetration into the dermis occurred when DMSO was the vehicle. After 24 hr of exposure, 12.1, 12.6 and 7.2 (mean percent of the dose) had penetrated into the dermis when MEOH, DMSO or water was the vehicle, respectively. DMSO is a dipolar aprotic solvent and is thought to displace bound water from skin, resulting in a looser skin structure [20]. The penetrant presumably diffuses through the DMSO in the membrane. Alternatively, DMSO may alter the skin on contact, diffuse rapidly through it, increase skin permeability, and thereby promote the penetration of materials dissolved in it [21]. The structural alteration of epidermis induced by DMSO and its analogues is, to some degree, reversible [22]. The rationale for use of DMSO was: 1) to enhance movement of toxin across the membrane of skin, which can then simulate the effects of abrasion on dermal penetration [23], and 2) to determine the extent of skin penetration if laboratory worker involved in extracting microcystin accidentally came in contact with

microcystin in the presence of a solvent.

The low total recovery (50 - 89 %) of the microcystin could be due to difficulty in extracting microcystin from skin sections and break down of microcystin in soap (epidermal surfaces of the skin were washed with 1 : 10 dilution of soap and water).

HPLC analysis of receptor fluid and skin extract from skin discs dosed with vehicle (MEOH, DMSO and water) showed solvent peaks only. This indicated that if constituents of skin leached out of skin into the receptor fluid, these constituents either had longer retention times (> 20 min) or else they were not strong UV absorbers.

Chromatographic analysis of receptor fluid and skin extracts from the human skin discs dosed with microcystin dissolved in different vehicles showed no peaks other than the solvent peaks and microcystin peak Fig.3. These results indicate microcystin was not metabolized by excised human skin. Another possibility is that microcystin was metabolized but the metabolites had longer retention times (> 20 min), or were not strong UV absorbers.

Microcystin is a lipophilic compound which does not readily penetrate into aqueous receptor fluid. It has been reported that in vitro penetration of compounds mimics more closely the in vivo skin penetration when amount recovered from dermis and receptor fluid is added together rather than calculating in vitro skin absorption based on recovery of penetrant from receptor fluid only. Accumulation of the compound in the dermis in in vitro system is possibly due to lack of functional microcirculation

which plays an important role in in vivo system.

CONCLUSION

Topical application of microcystin to excised human skin results in penetration of microcystin into skin layers and receptor fluid which bathes dermal surface. In vitro skin penetration was calculated by summing microcystin recovered in the dermis and receptor fluid. After 24 hr of topical exposure, approximately 13 percent of the dose (50 μ g microcystin) had penetrated the skin when methanol or DMSO was the vehicle. When water was the vehicle 9 percent of the dose penetrated the skin after 24 hr of topical exposure.

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Table 1. Effect of vehicle on distribution of microcystin following topical application to excised human skin.

Time of exposure (hr)	Component	VEHICLE		
		Methanol	DMSO	Water
4	Skin Surface	61.53 ± 11.95	51.60 ± 0.83	41.60 ± 8.66
	Stratum Corneum	0.22 ± 0.22	0.64 ± 0.58	0.68 ± 0.27
	Epidermis	1.27 ± 0.52	2.18 ± 2.10	6.63 ± 2.41
	Dermis	2.16 ± 1.03	2.75 ± 1.06	0.9 ± 0.32
	Receptor Fluid	0.24 ± 0.24	0.07 ± 0.01	0.26 ± 0.08
	Recovery	67.14 ± 9.17	57.25 ± 2.73	50.07 ± 7.69
12	Skin Surface	58.65 ± 7.92	47.68 ± 1.90	57.60 ± 5.11
	Stratum Corneum	2.0 ± 1.1	4.20 ± 1.14	1.44 ± 0.58
	Epidermis	3.2 ± 1.9	2.89 ± 0.95	5.28 ± 3.19
	Dermis	3.44 ± 0.72	10.20 ± 4.12	4.68 ± 2.2
	Receptor Fluid	0.26 ± 0.19	0.16 ± 0.13	0.25 ± 1.04
	Recovery	73.46 ± 2.84	65.18 ± 6.98	73.34 ± 2.55
24	Skin Surface	49.98 ± 9.41	72.76 ± 1.45	59.62 ± 3.18
	Stratum Corneum	1.22 ± 0.34	1.06 ± 0.54	2.04 ± 0.84
	Epidermis	0.78 ± 0.19	2.30 ± 1.77	2.33 ± 0.98
	Dermis	12.08 ± 2.08	12.63 ± 8.57	7.21 ± 2.95
	Receptor Fluid	0.49 ± 0.09	0.73 ± 0.25	1.49 ± 0.10
	Recovery	61.94 ± 8.02	89.49 ± 9.23	71.24 ± 1.86

^aResults are expressed as percent of the dose (mean ± SE)

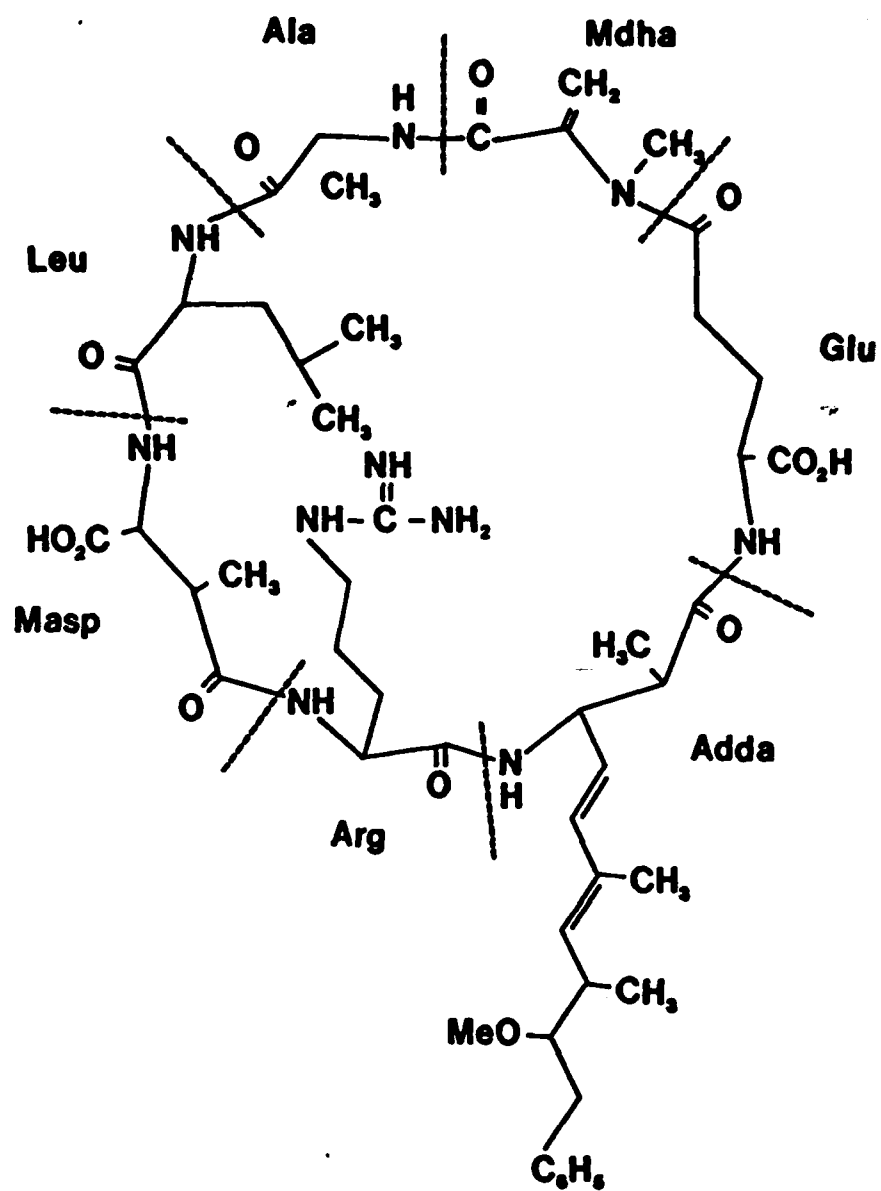


Figure 1. Structure of Microcystin

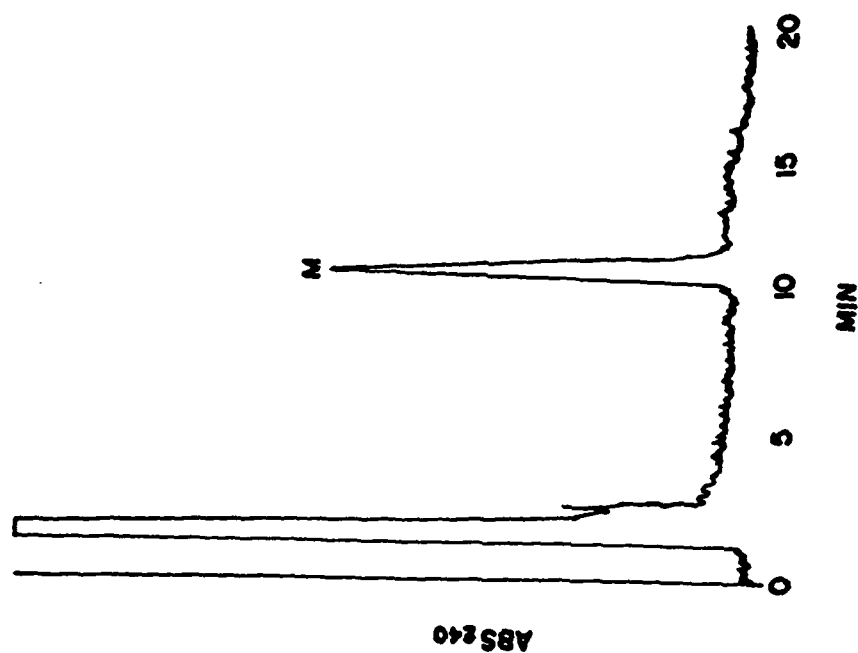


Figure 2. High performance liquid chromatography of purified microcystin (2.5 ug), M = microcystin peak. Chromatographic conditions were: reverse phase C18 uBondapak column, 10 μ m, 3.9 mm x 36 cm; mobile phase - 0.1 M ammonium acetate (pH 4-6): acetonitrile (75:25, v/v); flow rate - 0.5 ml/min; wavelength was monitored at 240 nm.

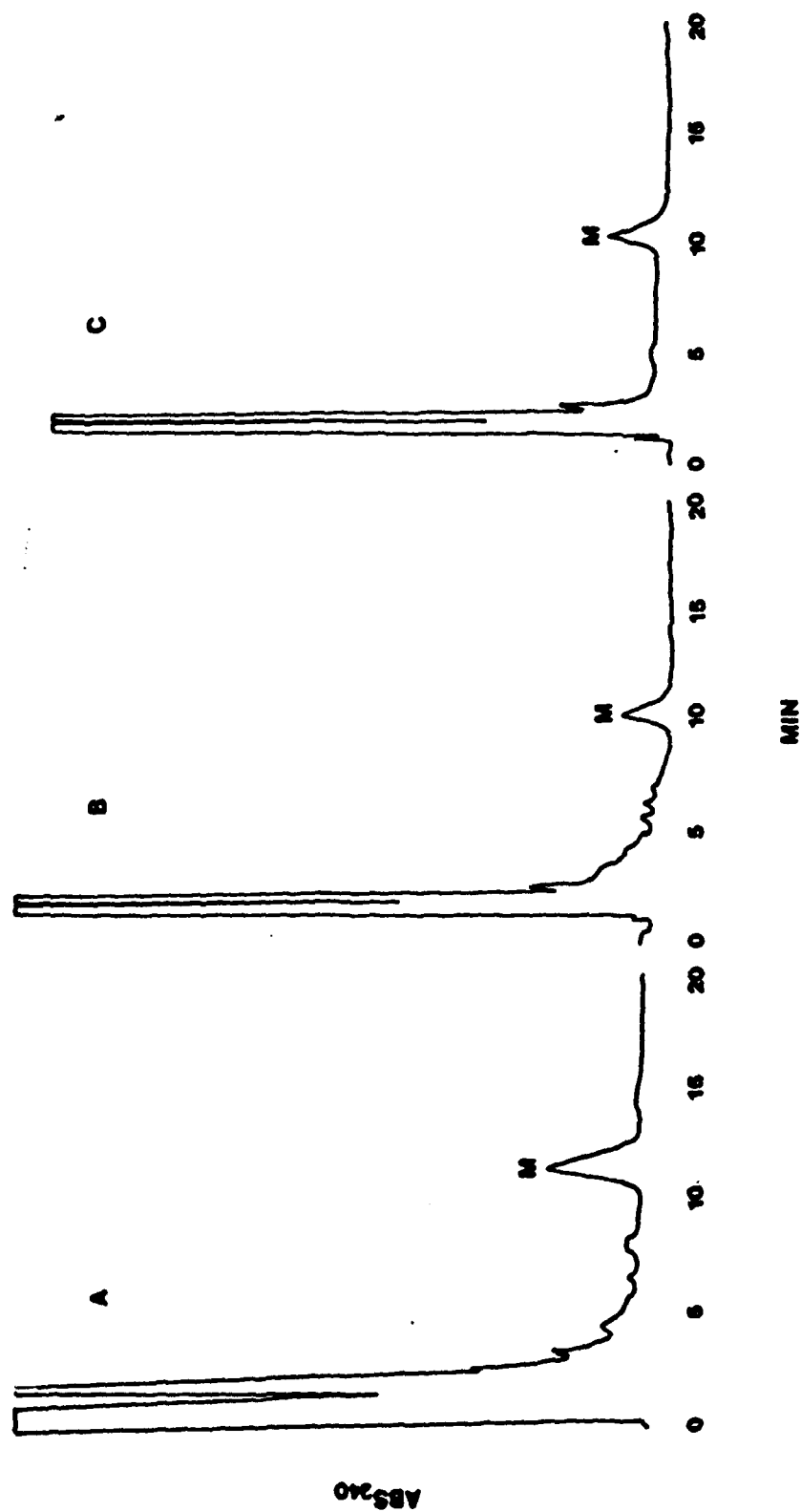


Figure 3. HPLC chromatographic analysis of receptor fluid from human skin dosed with microcystin dissolved in, A. 50 µl of DMSO, B. 50 µl of water and C. 50 µl of methanol. M = microcystin. Chromatographic conditions were same as described in figure 2.

In Vitro penetration of tritium labelled water (THO) and [³H]PbTx-3 (a red tide toxin) through monkey buccal mucosa and skin.

M.Mehta, B.W. Kemppainen and R.G. Stafford.

Department of Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, AL 36849.

STATEMENT OF PROBLEM

The primary purpose of this study was to determine the penetration of tritiated water (THO) and [³H]PbTx-3 through monkey buccal mucosa and skin. According to the contract, these studies were planned to be conducted in excised human buccal mucosa, but due to difficulty in obtaining human buccal mucosa, it was decided to conduct the studies in excised monkey tissue. Monkey tissue was used because the animal models most predictive of percutaneous absorption in man are pig and monkey (Ronald C. Wester and Patrick Noonan, 1980). Penetration of THO through monkey buccal mucosa and skin was studied so that the permeability of monkey buccal mucosa could be compared to the permeability of dog and pig buccal mucosa (William R. Galey et al., 1976). Penetration of THO through human skin was also determined so as to compare the results of this study with the published values for human skin. Distribution of [³H]PbTx-3 into

various layers of monkey buccal mucosa and skin was determined because the dermis acts as an important barrier to the penetration of lipophilic compounds through excised skin.

A portion of this work was done last year but has been included in this report because: i) the formula used to calculate permeability coefficient (K_p) was previously interpreted incorrectly, ii) penetration of [3H]PbTx-3 was previously determined only for a single time period (e.g., 12 or 24 hr), iii) tissue discs were not sliced and extracted to determine distribution into various layers. Therefore, further work was done this year to determine these additional parameters.

BACKGROUND

PbTx-3 is one of a group of brevetoxins produced by a marine dinoflagellate Ptychodiscus brevis. Blooms of this dinoflagellate are referred to as red tide. This algae has a soft outer covering which ruptures in the surf and results in the release of toxins. Blooms of P. brevis are responsible for massive fish kills in the Gulf of Mexico and along the Florida coast [1]. The red tides are also implicated in human intoxication resulting from ingestion of contaminated shellfish or inhalation of seaspray aerosols. Persons swimming in 'red tides' may experience eye and skin irritation and itching. Ingestion of shellfish contaminated with brevetoxins causes nausea, cramps, paresthesia of the lips, face and extremities, occasionally weakness and

difficulty in movement, even apparent paralysis, seizures and coma [2].

The lipid soluble toxins produced by P. brevis are of two types, one of which is hemolytic and the other neurotoxic [3]. The neurotoxin has a unique structure of a cyclic polyether (Fig. 1) with a molecular weight of 898 [4]. The in vivo experimental effects of P. brevis are both gastrointestinal and neurological in nature [5]. The LD₅₀ of PbTx-3 for female mice is 4 ug/kg, i.p. [6].

The primary purpose of this work was to determine the penetration of PbTx-3 through monkey buccal mucosa and skin. Due to the extremely toxic nature of the compound, these studies can not be conducted in human beings. Comparative studies have indicated that skin absorption in monkeys approximates skin absorption in humans [7,8,9]. Accordingly, buccal mucosa and skin from rhesus monkeys was used to assess the penetration of tritiated water (THO) and PbTx-3. In addition, the penetration of THO through human skin was determined so that we could compare our results to published values for humans. To our knowledge,

no report has appeared describing the penetration of brevetoxin through buccal mucosa. The degree of correlation between in vivo and in vitro skin penetration depends on the chemical properties of the penetrant and methodology. In general in vivo skin penetration has been found to correlate well with in vitro skin penetration [10,11,12].

MATERIALS AND METHODS

THO was generously provided by Dept. of Radiological Safety, Auburn University, AL. [^3H]PbT-3 was purchased from Dr. D.G. Baden (University of Miami, FL). The specific activity was 12.3 - 14.3 Ci/mMol. The radiochemical purity of [^3H]PbTx-3 was determined with reverse phase high pressure liquid chromatography (HPLC) prior to each experiment. HPLC was performed with a unit from Waters Associates (Milford, MA) equipped with a variable wavelength detector. For radiochromatographic analysis of [^3H]PbTx-3, a radioactive flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL) was attached to HPLC system. The scintillation cocktail was Flo-scint (Radiomatic). The HPLC column was a 10 μm C_{18} uBondapak steel column (2 mm x 36 cm). The eluant was [methanol : water (85:15)] pumped at rate of 0.5 ml/min and ultraviolet (UV) absorbance was monitored at 215 nm. The purity of this [^3H]PbTx-3 was determined to be more than 99 percent. Retention times of [^3H]PbTx-3 and contaminant peak were 4.0 to 4.2 min and 1.0 to 1.3 min, respectively.

Rhesus monkey buccal mucosa and skin were obtained from Wisconsin Regional Primate Research Center (Madison, WI), Delta Primate Center (Covington, LA), California Regional Primate Research Center (Davis, CA) and Washington Regional Primate Research Center (Seattle, WA). The monkeys were sacrificed as part of other ongoing studies. Collected tissues were packaged with wet ice and shipped by overnight delivery. Tissues were used immediately on arrival. Human cadaver abdominal skin was obtained

from local hospitals and was stored at 4°C for up to 5 days prior to use. Previous studies [13] have shown that the permeability of skin did not change significantly when stored by this method for up to 10 days. Split thickness human skin (700 - 800 μm) was prepared with a Padgett Electro Dermatome (Padgett Instruments, Kansas City, MO).

Loose fat and connective tissue was removed from the inner surface (dermal side) of monkey buccal mucosa and skin. Excised tissue discs had a diffusible surface area of 2.8 cm^2 and were mounted on static teflon diffusion cells, Fig. 2, [14]. The donor and receptor chambers of diffusion cells were filled with Hanks's Balanced Salt Solution with HEPES buffer and gentamicin (HBSS). Several groups of assembled diffusion cells were incubated at 4°C for 15 hr in order to fully hydrate the tissue discs prior to penetration experiments. The receptor fluid was bubbled with O_2 : CO_2 (95:5) to maintain the viability of the tissue discs [15]. One group of the buccal mucosal discs was placed in between two layers of large pore nylon membranes (Cole Parmer, Chicago, IL) in order to determine if it was necessary to use additional support for the delicate buccal mucosa. One group of monkey skin discs was also placed in between the nylon membranes to determine if there was hindrance to penetration due to the nylon membranes. At time 0, HBSS on outer surfaces was replaced with dose, 0.8-1.0 μCi of THO or 371-412 ng of [^3H]PbTx-3 (5-7 μCi) dissolved in 2 ml of water. The epidermal surfaces were occluded for the entire exposure period (15 min - 24 hr). The diffusion cells were

incubated in an environmental chamber at 37°C in order to maintain them at a constant temperature. Sequential samples (50 μ l) were collected from the dermal side (receptor fluid) every 15 min during the first hr, every 30 min during time 1 to 4 hr, every one hr during 4 to 24 hr). Fresh HBSS was added to the receptor chambers after each sample in order to replace the amount that was removed. At the end of each experiment, the dose left on the epidermal surface was collected and measured. Epidermal surfaces were gently washed with cotton swabs moistened with soap and water (1:10 dilution) in order to determine the amount of dose remaining on the epidermal surface. Cotton swabs and tissue discs from the diffusion cells dosed with THO or [3 H]PbTx-3 were placed in vials containing 10 ml of water or methanol, respectively. Extraction of the radiolabeled compounds from the tissue was facilitated by sonicating the vials for 10 minutes. Radioactivity in the receptor fluid, skin wash, skin extracts and dose left on the epidermal surface was assessed by mixing aliquotes of each sample with scintillation cocktail (Hydroflour, National Diagnostics, Manville, New Jersey) and then counting each vial with a Liquid Scintillation Analyzer (Packard Model 1500, Tri-carb, Sterling, VA).

The distribution of [3 H]PbTx-3 into the layers of buccal mucosa (epithelium and lamina propria) or skin (epidermis and dermis) was determined. Tissue discs were immediately frozen (-20°C) at the end of exposure period and sectioned with a microtome (Bauch & Lomb, Optical Co., Rochester N.Y.) at a

setting of 25 μ m. Tissue slices were extracted in methanol and radioactivity was determined with standard liquid scintillation methods.

The permeability coefficient (K_p) for THO was calculated by dividing the net total transfer of compound during each time interval by the product of the amount of the compound in the donor side solution and the surface area of the tissue disc available for diffusion. For each treatment group, K_p (mean \pm SD) was calculated from all time intervals. K_p is related to the total net flux by the equation:

$$K_p = J / \Delta C \times A$$

where J = net flux of the compound; ΔC = the concentration difference across the tissue disc; A = the surface area of the tissue disc exposed to the compound.

The stability of [3 H]PbTx-3 in the receptor fluid (HBSS) was evaluated by mounting Teflon discs, instead of tissue discs, on one or two diffusion cells in each experiment. The receptor fluid was dosed with a fraction of the dose of [3 H]PbTx-3 prepared for the respective experiment. The diffusion cells containing Teflon discs were incubated at 37°C along with the diffusion cells containing tissue discs. Receptor fluid samples from the diffusion cells containing teflon or tissue discs were treated in the same manner. The ability of excised monkey buccal mucosa and skin to metabolize [3 H]PbTx-3 was determined. At the end of each experiment, tissue discs dosed with [3 H]PbTx-3 were extracted with methanol and radiochemical analysis was done with HPLC.

Similarly, aliquotes from receptor fluid were also analyzed.

Statistical analyses of data was performed by comparing means using Student's t test. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

The permeability coefficients for buccal mucosa and skin are listed in Table 1. The buccal mucosa was generally in the range of 1000 - 1200 μM thick. The thickness of the monkey skin and human skin was in the range of 700 - 800 μM . In order to facilitate comparison, the K_p of skin and buccal mucosa have been adjusted to a thickness of 750 μM thick, as described by Galey et al. [16]. The permeability of excised human skin was determined to correlate favorably with other reports [17]. Hydration of human skin (prior to penetration experiment) almost doubled the permeability of human skin to THO. Hydration has the effect of swelling the cells and reducing the density of their structure and their resistance to diffusion. Hydration of the stratum corneum can increase diffusion constants up to 10 fold [18]. No significant difference ($P > 0.05$) was observed between permeability of hydrated human skin and hydrated monkey skin. Percutaneous absorption in rhesus monkey is generally similar to that in man [19,20,21].

Monkey buccal mucosa was determined to be eight times more permeable to THO than monkey skin. The larger K_p of monkey buccal

mucosa than monkey skin for THO is in agreement with the generally held view that buccal mucosa is more permeable than skin. The greater permeability monkey buccal mucosa is probably due to the lack of keratinized epithelium [22]. Nylon membranes did not alter the penetration of THO through monkey buccal mucosa or skin ($P > 0.05$). It was concluded that it is not necessary to provide additional support to the delicate buccal mucosa.

Preliminary analysis of the accumulation of [^3H]PbTx-3 in receptor fluid bathing the inner surfaces of monkey buccal mucosa and skin indicated a long lag phase and slow penetration rate (see Figure 3). These findings are consistent with other reports where diffusion of lipophilic penetrants into receptor fluid is minimal due to limited solubility in the aqueous fluid [23]. Wannemacher et al. [24] have reported that PbTx-2 (a related compound) has poor solubility in water ($< 100 \text{ ug/L}$). This has been further confirmed by Dr. D. Baden (personal communication) who estimates the solubility of PbTx-3 in water to be 100 ug/L . Therefore, instead of determining the K_p of buccal mucosa and skin to [^3H]PbTx-3, the distribution of radioactivity in monkey buccal mucosa and skin following topical application of [^3H]PbTx-3 was determined (Table 2). At each time interval, the largest portion of the applied dose was recovered from the tissue surface (epithelial or epidermal). As early as 15 min after dosing, [^3H]PbTx-3 had penetrated into the epithelium/epidermis and lamina propria/dermis. No uniform pattern was observed for penetration into the epithelium/epidermis. The percent of the

dose that penetrated into the lamina propria/dermis increased with time (15 min to 24 hr). After 24 hr, 34 and 13 percent of the dose had penetrated into the lamina propria and dermis, respectively. [^3H]PbTx-3 recovered from receptor fluid was less than 2 percent for all the time intervals studied, e.g. 15 min to 24 hr.

In contrast to the large amount of PbTx-3 recovered from the lamina propria/dermis, after 24 hr, only 2 and 8 percent of THO was residing within the buccal mucosa and skin, respectively. It has been reported that during in vitro studies the dermis acts as an artificial reservoir for lipophilic compounds but not for hydrophilic compounds [12]. In in vivo studies the dermal microvasculature transports the penetrant to systemic circulation which reduces the accumulation of the penetrant in the dermis. Hawkin's et al. [12] and Kemppainen et al. [25] have determined that in vitro skin penetration of lipophilic compounds correlates better with in vivo skin penetration when in vitro values are calculated by summing the amount of penetrant in dermis and receptor fluid.

The stability of [^3H]PbTx-3 in receptor fluid (HBSS) was determined at 37°C. The [^3H]PbTx-3 preparation breaks down at the rate of approximately 10 % during 24 hours. Radiochromatographic analysis of samples from the skin surface, skin extract and receptor fluid indicated that between 85 and 95 % of the radioactivity in each sample was associated with [^3H]PbTx-3. The remaining activity (5 to 15 %) in each sample was associated with

a breakdown product which had a retention time of 1 to 1.3 min. These results are consistent with the hypothesis that PbTx-3 was not metabolized by excised monkey buccal mucosa or skin.

In conclusion, PbTx-3 readily penetrates into the layers of buccal mucosa/skin, but probably due to its low solubility does not diffuse into receptor fluid. Therefore, when using an in vitro method to assess the penetration of lipophilic compounds through tissue it is important to determine the distribution into layers of buccal mucosa and skin.

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Table 1. Permeability Coefficients of buccal mucosa and skin^a to tritiated water (THO)

Tissue	Kp cm/hr, mean \pm SD X 10 ⁻³
Hydrated MS (11) [6] ^b	0.77 \pm 0.57
Hydrated MSN (7) [3]	0.65 \pm 0.24
Hydrated Monkey BM (8) [3]	6.15 \pm 2.57
Hydrated Monkey BMN (4) [3]	5.90 \pm 1.94
Human Skin (not hydrated) ^c (7) [3]	0.47 \pm 0.15
Hydrated Human Skin (7) [3]	0.88 \pm 0.42

^a MS = Monkey skin, MSN = Monkey skin supported by nylon, BM = Buccal Mucosa, BMN = Buccal mucosa with nylon.

^b () total number of replicates, [] number of donor individuals.

^c Hydrated or not hydrated refers to whether or not tissues were incubated (4°C) in Hanks Balanced Salt Solution prior to penetration study.

Table 2. Distribution of [³H] PbTx-3 in monkey buccal mucosa and skin after topical application

Time After Dosing	Barrier	(n) [N]	Percent of dose (Mean \pm SD) APPLIED					Total Recovery
			Skin Surface ^b	Epithelium/ Epidermis	Lamina propria/ Dermis	Receptor Fluid		
15 Min	BM	(3) [3] ^a	87 \pm 4.4	0.34 \pm 0.12	1.3 \pm 0.13	0.02 \pm 0.01	95 \pm 4.5	
	MS	(3) [3]	83 \pm 3.6	1.1 \pm 0.11	1.5 \pm 0.14	0.006 \pm 0.004	92 \pm 6	
2 hr	BM	(3) [3]	91 \pm 7	0.62 \pm 0.05	3.5 \pm 0.77	0.02 \pm 0.01	95 \pm 5.6	
	MS	(3) [3]	95 \pm 5	0.74 \pm 0.46	1.5 \pm 0.60	0.01 \pm 0.01	97 \pm 7.6	
4 hr	BM	(3) [3]	84 \pm 6.4	1.4 \pm 0.70	6.4 \pm 1.7	0.43 \pm 0.31	92 \pm 7	
	MS	(3) [3]	91 \pm 9	1.3 \pm 0.8	3.3 \pm 0.55	0.02 \pm 0	95 \pm 8.5	
12 hr	BM	(3) [3]	71 \pm 11	0.72 \pm 0.37	2.2 \pm 7	1.3 \pm 0.62	96 \pm 5.5	
	MS	(5) [4]	90 \pm 10	1.2 \pm 0.34	4.8 \pm 1.9	0.10 \pm 0.03	96 \pm 6.4	
24 hr	BM	(6) [4]	54 \pm 21	2.4 \pm 0.92	33 \pm 9.0	1.9 \pm 1.1	92 \pm 3.5	
	MS	(6) [4]	79 \pm 18	0.88 \pm 0.28	12 \pm 8.4	0.38 \pm 0.21	93 \pm 4.9	

^a = (n) = Total number of replicates. [N] = total number of individuals.^b = Includes skin wash \pm Dose left on epithelial/epidermal surface.

BM = Buccal Mucosa, MS = Monkey Skin.

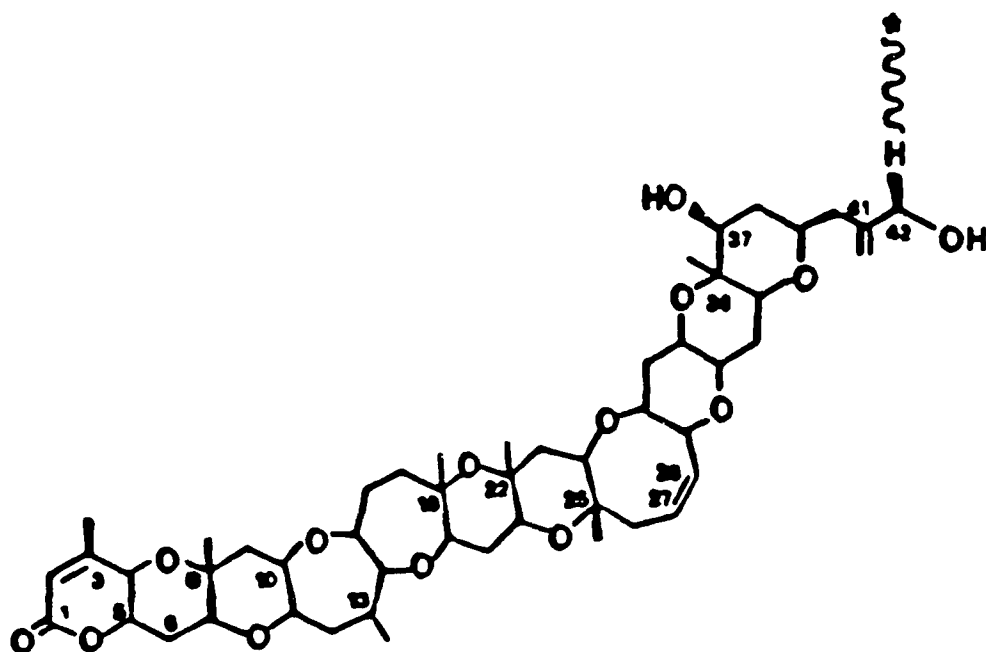


Figure 1. Structure of tritium labeled PbTx-3.

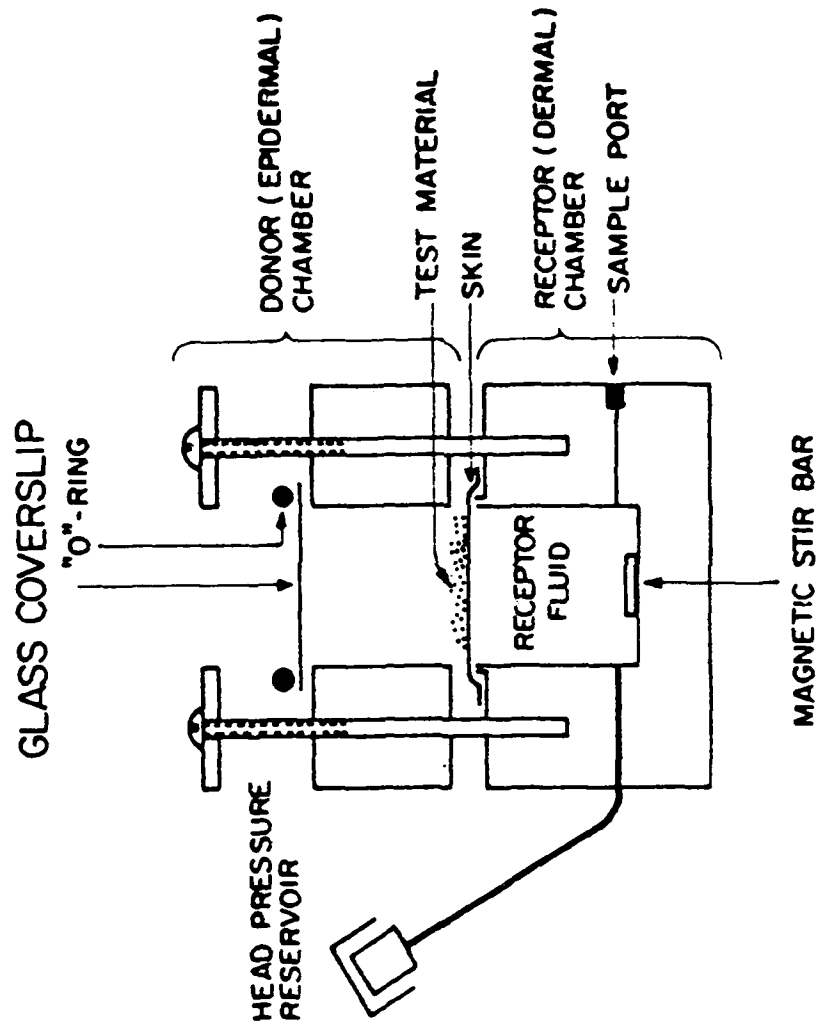


Figure 2. Diagram of static diffusion cell used for buccal mucosa and skin penetration studies.

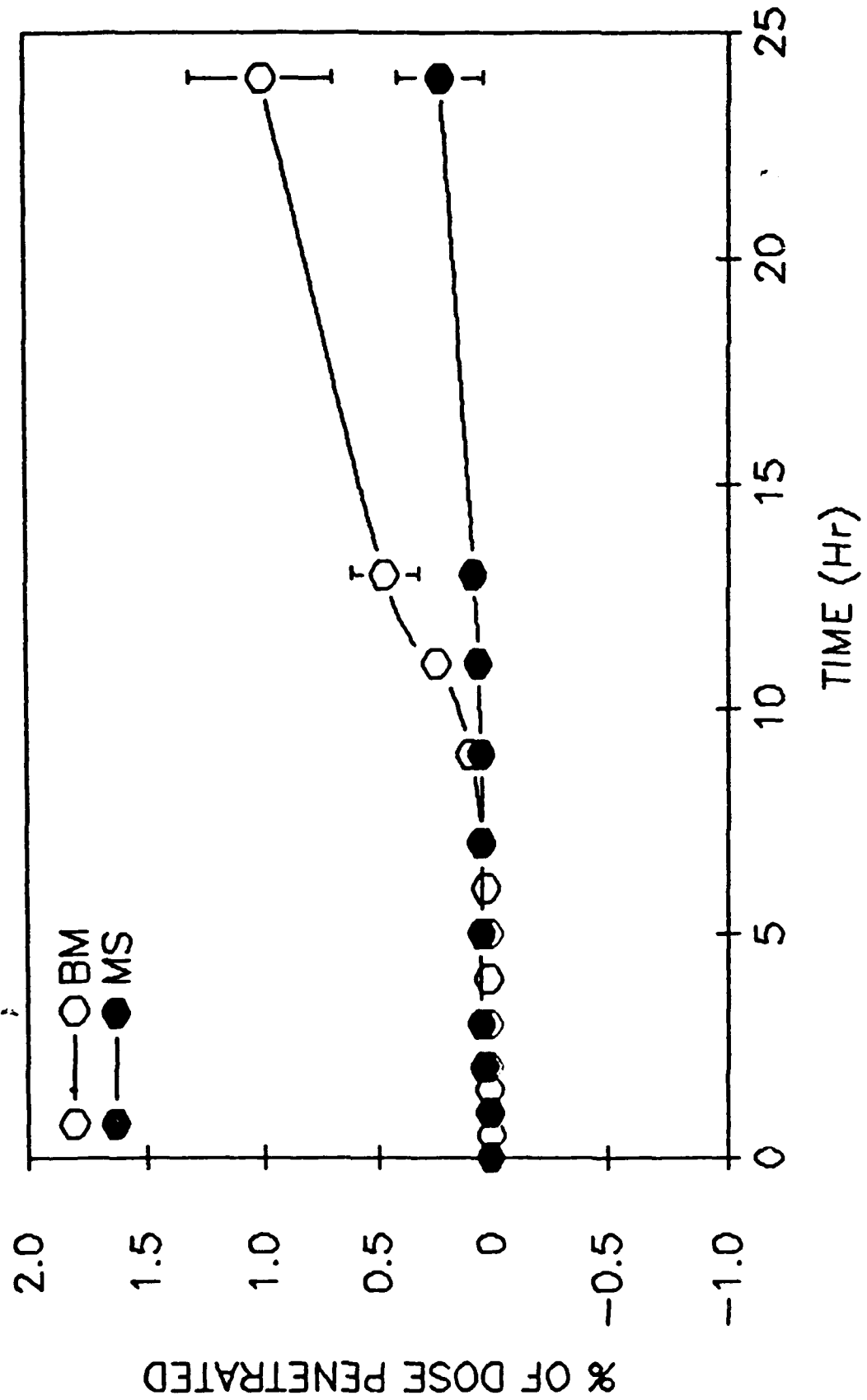


Figure 3. Penetration of brevetoxin ($[^3\text{H}]\text{PbTx-3}$) through excised monkey buccal mucosa and skin. Results expressed as percent dose recovered in receptor which bathed dermal (inner) surface of tissue. See text for detailed description of methods.

PENETRATION OF LYNGBYATOXIN A THROUGH GUINEA PIG SKIN IN VITRO

Robert G. Stafford

Barbara W. Kemppainen

Meena Mehta

STATEMENT OF THE PROBLEM

The purpose of this work was to determine the amount of percutaneous absorption of low molecular weight (M.W.) toxins through excised human and guinea pig skin. The toxin currently being studied is lyngbyatoxin A (teleocidin A).

BACKGROUND

A blue-green alga called Lyngbya majuscula Gomont is common in the tropical Pacific and Indian Oceans. Known since 1912 in Hawaii, L. majuscula was familiar to inhabitants as a source of irritation upon contact in fishing nets or while swimming. The deaths of horses eating the alga have occurred in Ceylon. In 1958 a massive outbreak of dermatitis at Kailua, Oahu, Hawaii was attributed to L. majuscula. L. majuscula is common on many beaches but is not toxic in all cases (1). Some varieties found in deep water were found to produce two toxins called debromoplysiatoxin and plysiatoxin (which were first isolated in the digestive tract of the sea hare Stylocheilus longicauda, [8,9]). Swimmer's itch was first reported in Japan in the summer of 1968 on Okinawa and was at first attributed to pollution from disposed war gases. Symptoms included an itching, burning rash, blisters and deep erosion of the skin. Hashimoto (5) suggested that L.

majuscula, was the cause of this dermatitis in Okinawa. In the summer of 1980, there was a severe outbreak of swimmer's itch on Oahu Island, Hawaii, and aplysiatoxin and debromoaplysiatoxin from L. majuscula were identified as the causative agents (7, 8, 10). Another toxin, lyngbyatoxin A, was found in L. majuscula gathered from shallow water at Kahala Beach, Oahu. All of these compounds cause erythema, blisters and deep desquamation (2). The structure of lyngbyatoxin A was determined to be similar to teleocidin B isolated from several Streptomyces strains (fungus-like bacteria). Teleocidin was isolated from Streptomyces medio-cidicus as a strong skin irritant and is a mixture of 93% teleocidin A and 7% teleocidin B (4). Lyngbyatoxin A and teleocidin B have an indole group and a nine membered lactam structure which has been termed an indolactam by Fujiki and coworkers. Lyngbyatoxin A and Teleocidin B have similar toxicities in mice, LD₁₀₀=0.03 mg/kg I.P. for lyngbyatoxin A or 0.22 mg/kg I.V. for Teleocidin B (2). Fujiki and coworkers using several different tests and screening certain compounds for tumor promoting activity found that dihydroteleocidin B was a very strong tumor promoter (3). Subsequently they found that aplysiatoxin, debromoaplysiatoxin, lyngbyatoxin A and teleocidin are all tumor promoters which bind to the same cell surface receptor as the original tumor promoter (12-O-tetradecanoylphorbol-13-acetate, TPA) and cause similar biological effects (4).

RATIONALE USED IN THIS STUDY

The purpose of these studies are to determine the extent to which these toxins penetrate the human skin. Due to the extreme toxicity of these compounds it is not feasible to use human subjects. Instead, in vitro techniques are being used to measure the penetration of these compounds through excised skin. In this study two types of excised skin are being compared, excised human skin and excised guinea pig skin. To date, two guinea pig experiments have been performed and are presented here.

MATERIALS AND METHODS

Receptor Fluid: The receptor fluid was HEPES buffered Hanks Balanced Salt Solution (HHBSS, Gibco Laboratories Life Technologies Inc., Grand Island, NY) with Gentamicin sulfate (Sigma Chemical Co., St. Louis, MO).

Recipe for HHBSS: 100 ml 10x HBSS concentrate mixed with 850 ml grade I water.

Add 4.7 ml 7.5% NaCO_3

Add 10 ml HEPES Buffer (1 M)

Add 1 ml Gentamicin sulfate (50 mg/ml)

pH to 7.3 - 7.4 with HCl or NaOH

Bring up volume to 1 Liter

Filter using sterile filter

Preparation of skin: Guinea pigs weighing between 986 and 993 grams were sacrificed using carbon dioxide. The ventral surface of the animal was shaved using animal clippers. Care was taken not to injure the skin. The shaved skin was removed using scissors and rinsed to remove loose hair. The skin was placed in the

refrigerator in a stainless steel pan on a paper towel soaked with HHBSS (epidermal side up) until used, not more than 24 hours later. Disks of full thickness abdominal skin were cut and the thickness measured by means of a spring loaded micrometer (Mitutoyo, Japan).

Assembly of the cells: Receptor fluid and stir bars were added to the teflon diffusion cells. The area of the skin surface was 2.5 cm². Skin disks were placed on the cells and the assembly was completed. Receptor fluid was added through the vent to check for leaks (see Figure 1). The cells were placed on a magnetic stirrer base and placed in 37°C environmental chamber for approximately an hour to equilibrate. The magnetic stirring base keeps the receptor fluid stirred.

Dosing and incubation of the cells: The treated cells were dosed with 50 µg lyngbyatoxin A dissolved in 25 µl of DMSO (2 µg/µl). The cells were incubated in the chamber for varying periods of time ranging from 1 to 48 hours. The control cells were dosed with vehicle (25µl DMSO, HPLC grade, Aldrich Chemical Co., Inc., Milwaukee, WI) in order to determine if skin or receptor fluid extracts contained compounds which co-elute with lyngbyatoxin A.

Standard preparation: The lyngbyatoxin A (2 µg/µl) was diluted to 0.05 µg/µl in DMSO. One aliquot was frozen for use as a standard, another was left at room temperature to determine if the lyngbyatoxin would break down at room temperature.

Disassembly of the cells: The surface of the skin was washed with 2 balls of glass wool (Fisher Scientific Co., Norcross, GA) dampened with soapy water (1% Liqui-Nox laboratory detergent,

Alconox, Inc., NY,NY) and 2 swabs (glass wool) dampened with distilled water. This was done to recover the lyngbyatoxin A remaining on the skin surface. The skin was removed and mounted using OTC embedding compound (Miles Inc., Elkhart, IN) on plastic blocks (Thomas Scientific Co., Inc., Swedesboro, NJ) and frozen at -20°C .

Extraction of the receptor fluid: The receptor fluid was placed into a 5 ml Mixxor liquid/liquid extractor apparatus (Lidex Technologies, Inc., Bedford, Mass.) and 1ml Chloroform (Fisher Certified) was added. The phases were mixed, allowed to separate, and the chloroform phase removed. The receptor fluid was extracted in this way at least 3 times or more depending upon the amount of emulsification that occurred. The chloroform extracts of the receptor fluid were dried down under a gentle stream of nitrogen and then reconstituted to 200 μl with chloroform.

Skin sectioning: The frozen skin was sectioned parallel to the skin surface using a microtome (Bausch and Lomb Optical Co., Rochester, NY). The first vial contained stratum corneum, the first 15 μ . The second vial contained epidermis, from 15 - 100 μ . The third vial contained the dermis (skin at a depth $> 100 \mu$) which was minced with scissors and added directly to the vial.

Extraction of the skin sections: Chloroform (2 ml) was added to each vial including the vial containing the swabs from washing the skin surface. The vials were sonicated for 10 minutes to facilitate extraction of lyngbyatoxin from the samples and placed in the freezer over night. The chloroform was removed from the skin sections and the vials rinsed out three times with chloro-

form. The dermis was sonicated twice and the vials were rinsed out. The chloroform extracts from the skin were dried down under a stream of nitrogen. Each extract was reconstituted to 200 μ l with chloroform, except the vials containing the dermis which would not dry down and instead formed a viscous yellowish liquid on the bottom of the vial. A quantity of chloroform was added to this liquid and the total volume measured with a Hamilton syringe. The glass wool swabs from the skin washes were extracted as described for the skin sections.

Interfering substances: Skin disks and receptor fluid which had not been dosed with lyngbyatoxin A or were dosed with the vehicle only (DMSO) were extracted to look for peaks which would co-elute with lyngbyatoxin A. Also cotton swabs and glass wool were extracted with chloroform. The bottom part of Table 1 shows the results of searching for interfering substances.

HPLC method: The high performance liquid chromatography system consisted of a Waters Model 510 pump, Model 712 WISP, Model 441 absorbance detector at 254 nm wavelength and a Model SE-120 strip chart recorder. The column consisted of a Whatman Partisil 5 RAC II 4.6mm x 10cm column (silica, 5 μ particles). The mobile phase was changed slightly to improve the separation between an interfering peak and the lyngbyatoxin A peak. The original mobile phase consisted of 85% hexane, 10% chloroform and 5% 2-propanol (HPLC Grade, Fisher Scientific Co.). The modified mobile phase consisted of 90% hexane, 6.7% chloroform and 3.3% 2-propanol. The retention time for the lyngbyatoxin A peak using the new mobile phase varied between 14 and 21 minutes.

Quantitation of lyngbyatoxin A: Quantitation of the lyngbyatoxin A was performed by xeroxing the chromatograms, drawing an appropriate baseline and cutting the peaks out. In every case the standard lyngbyatoxin A peak was xeroxed on the same sheet of paper as the samples. The peaks were weighed on a Cahn Model 27 Automatic Electrobalance (Cahn Instruments, Cerritos, CA). Calculations were performed in the following manner:

Response Factor = Lyng. A std amount / Lyng. A std peak weight

Lyng. A sample amount = Lyng A peak Wt sample X Response Factor

In cases where there was a wide difference in peak weights, two different standard amounts of Lyngbyatoxin A were injected and linear regression was used to determine the sample amounts.

Summary of experiments:

First Experiment

Guinea pig weight: 992.7 gm, male

Average skin thickness: 859 μ Sd=92

A. Skin dosed with lyngbyatoxin A

two diffusion cells incubated for 4hr

two diffusion cells incubated for 24hr

two diffusion cells incubated for 48hr

B. Control skin (incubated for 48 hr)

one diffusion cell, nothing applied to epidermal surface

one diffusion cell, 25 μ l DMSO applied to epidermal surface

First Experiment (continued)

- C. Control diffusion cell with teflon disk in place of skin
5 μ l volume ($2\mu\text{g}/\mu\text{l}$) lyngbyatoxin A in DMSO added to
receptor fluid incubated for 48 hours.

Second Experiment

Guinea pig weight: 985.8 gm, male

Average skin thickness: 839μ SD=64

A. Skin dosed with lyngbyatoxin A

two diffusion cells incubated for 1hr

two diffusion cells incubated for 4hr

two diffusion cells incubated for 24hr

B. Control cells

two teflon membrane covered diffusion cells; 25 μ l
lyngbyatoxin ($2\mu\text{g}/\mu\text{l}$ in DMSO) added to receptor fluid,
incubated for 24 hr.

RESULTS AND DISCUSSION

Table 1 is a summary of a lyngbyatoxin stability study and results from the chromatography of components of a skin diffusion cell. Lyngbyatoxin dissolved in DMSO was fairly stable when stored at room temperature in the light (90% of original amount after 41 days). To determine the stability of lyngbyatoxin in HHBSS, two skin diffusion cells were set up with teflon sheets in place of skin. These diffusion cells were incubated for 24 hours with 50 μg of lyngbyatoxin A in the receptor fluid. The receptor fluid was extracted at the end of the incubation time. An average of 80.5% of the lyngbyatoxin A was recovered (see Table 1). In a

similar cell left for 48 hours only 11.9% of the lyngbyatoxin was recovered, however, extraction procedures were then altered to permit better efficiency of extraction and then 24 hour teflon cells were done. In order to see if lyngbyatoxin was stable overnight in chloroform, 25 μ l of a standard solution (5 ng/ μ l) of lyngbyatoxin was left at room temperature following injection into the HPLC. The next day the same standard was injected under the same conditions as the previous day and the results compared. The amount found was 164% of the previous day's results. The increased recovery was attributed to evaporation of the chloroform in the standard, resulting in a more concentrated sample. This illustrates that careful sample handling is needed. Care was taken to prevent occurrences of this sort. Chloroform extracts of the receptor fluid, skin, skin dosed with vehicle and cotton swabs were injected into the HPLC in order to find out if there were any interfering peaks present. Though many substances were detected, most did not interfere with lyngbyatoxin. As previously mentioned the mobile phase was changed somewhat to eliminate an interference from the skin. In the case of the cotton swabs, however, there was a peak with exactly the same retention time as lyngbyatoxin. The co-eluting peak could not be eliminated totally so glass wool was used as an absorbent material for washing the skin. Glass wool produced no interfering peaks.

Figure 2 is a set of chromatograms illustrating the identification of the lyngbyatoxin A peak in an epidermal skin sample. The retention time of lyngbyatoxin A in these chromatograms is

approximately 12 minutes (see Figure 2, Standard). An epidermal extract was injected and the peak labeled L was tentatively identified as lyngbyatoxin A. The standard was added to the sample in approximately a 1:1 ratio (v/v). The peak labeled L increased in size while all the other peaks became smaller (see Figure 2, Spiked Epidermal Extract).

The results of the two experiments can be found in Table 2. The results are expressed as average percent dose recovered from the samples \pm standard deviation. The percent dose equals the amount of lyngbyatoxin A recovered from sample divided by the amount of lyngbyatoxin A applied as the dose, times 100. As can be seen from Table 2, lyngbyatoxin A dissolved in DMSO seemed to penetrate rapidly into the skin, both the epidermis and dermis. After an hour the epidermis contained an average of 9.4% of the lyngbyatoxin A and the dermis contained 17%. In general these values increased over time. At 48 hours the epidermis contained 14% and the dermis contained 23% of the lyngbyatoxin A. The stratum corneum contained relatively little lyngbyatoxin A compared to the other skin layers, possibly due to the thinness of the layer (15μ). Whereas the epidermal layer was 85μ thick (15 to 100μ) and the dermis approximately 760μ thick (100 to 860μ). Lyngbyatoxin A could be found in the receptor fluid even after one hour. Because lyngbyatoxin A is a lipophilic compound, it remained in the dermis rather than diffusing into the receptor fluid. In the in vivo systems the capillaries pick up the absorbed compound and distribute it to the rest of the body. The in vitro skin disks have no functional capillaries to remove the

compound from the area. To better approximate in vivo skin absorption the amounts in the receptor fluid and the dermis should be added (6). While the early time periods had good recoveries, the 48 hour cells did not. One reason for the poor recovery is that the lyngbyatoxin may have been breaking down.

CONCLUSIONS

These experiments indicate that lyngbyatoxin A readily penetrates into excised guinea pig skin when DMSO is the vehicle. Also, in excised skin, the lyngbyatoxin apparently accumulates in the dermis perhaps due to the lack of functional capillaries and vasculature. Future plans include performing in vitro penetration experiments with lyngbyatoxin A on excised human skin.

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Table 1. Stability of Lyngbyatoxin and Interferences*

Stability of lyngbyatoxin A in solvents and receptor fluid (HHBSS)

	% Recovery
DMSO, R.T., 41 days	90
Chloroform, R.T., 24hr	164
HHBSS (teflon cell), 37°C, 24hr	81

Chromatography of chloroform extracts of components of skin diffusion system

CONTROLS (Chloroform Extracts)	Interfering Peaks
HHBSS (Receptor fluid)	No
Guinea pig skin	No
Guinea pig skin with DMSO (Vehicle)	No
HHBSS exposed to dermal side of skin	No
Cotton swab (For washing skin surface)	Yes
Glass wool (For washing skin surface)	No

*HPLC interferences caused by substances co-eluting with lyngbyatoxin A.

Table 2.
Disposition of Lyngbyatoxin A
in Excised Guinea Pig Skin

Time (hr)	Skin Surface	Stratum Corneum	Epidermis	Dermis	Receptor Fluid	Recovery
1	70 \pm 28 ^a	1.2 \pm 0.64	9.4 \pm 6.4	17 \pm 17	0.29 \pm 0.16	98 \pm 4.2
4	74 \pm 13	3.8 \pm 3.8	12 \pm 10	19 \pm 6.2	0.13 \pm 0.15	110 \pm 28
24	41 \pm 9.1	0.91 \pm 0.76	10 \pm 4.6	25 \pm 11	0.15 \pm 0.16	80 \pm 4.9
48	29 \pm 7.2	2.4 \pm 3.2	14 \pm 8.8	23 \pm 4.2	0.03 \pm 0.04	68 \pm 15

^aExpressed as percent dose (mean \pm standard deviation).

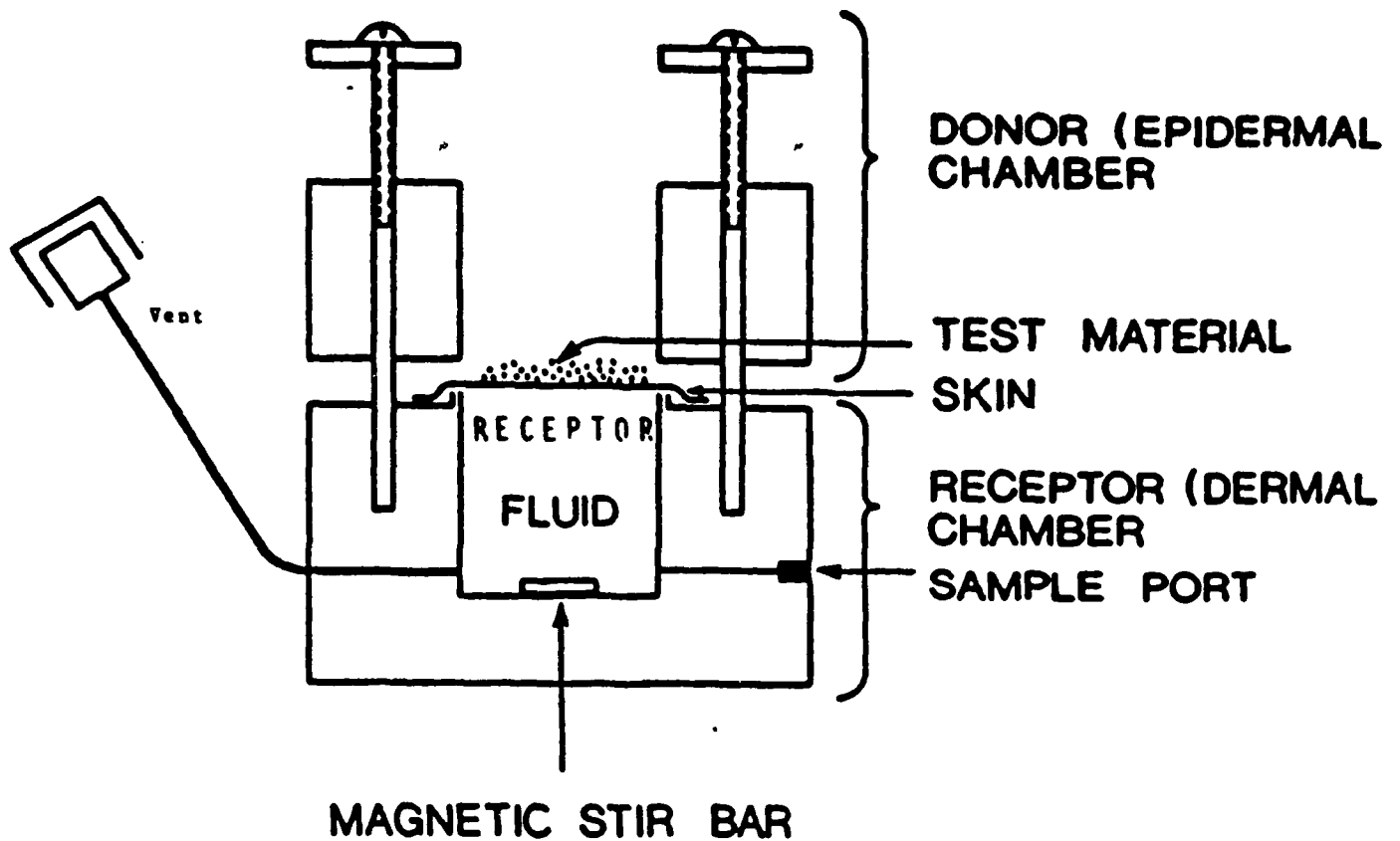


Figure 1. Static Teflon Diffusion Cell

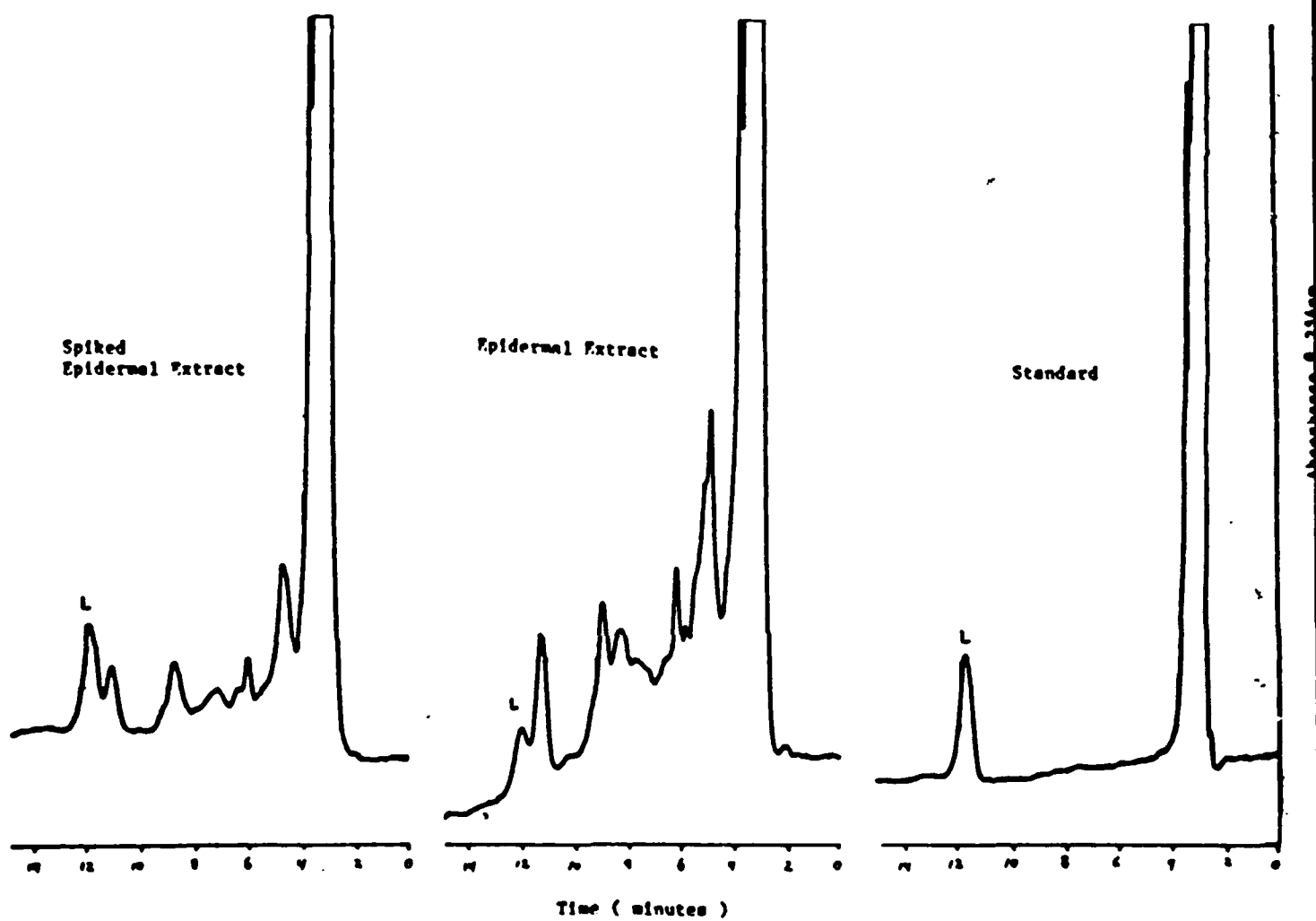


Figure 2. Three chromatograms illustrating the identification of lyngbyatoxin A in a skin sample. L identifies the lyngbyatoxin peak in each chromatogram.

PARTITION COEFFICIENTS FOR
[³H]PBTX-3, MICROCYSTIN AND LYNGBYATOXIN A

Robert G. Stafford

Barbara W. Kemppainen

Meena Mehta

STATEMENT OF THE PROBLEM

Partition coefficients (Log P) can be used to predict many properties of organic molecules such as their adsorption (e.g., binding to proteins), biological uptake, distribution and storage (1,4). The purpose of this contract is to determine the percutaneous penetration of low molecular weight toxins of military interest. The Log P values of the low molecular weight compounds might explain differences in the rates of absorption of these compounds. A literature search was unsuccessful in discovering values for these compounds. The purpose of this study is to determine the Log P of [³H]PbTx-3, microcystin and lyngbyatoxin A.

BACKGROUND

The logarithm of the oil/water concentration ratio, otherwise known as the partition coefficient (Log P), is a measure of the solubility of a compound in two immiscible solvents. Most frequently one solvent is aqueous and the other is 1-octanol(1,2,4). The percutaneous absorption of a compound is influenced by the solubility of the compound in the lipid and

aqueous portions of the skin (3). When comparing in vitro to in vivo penetration experiments, the Log P_{octanol} value is important. In general, as the Log P increases, the ratio of the in vivo to in vitro penetration increases.

The octanol/water partition coefficient is generally easily determined by dissolving a known quantity of the solute in a flask containing water and octanol (or another immiscible solvent) and shaking until equilibrium is reached. The phases are centrifuged to break the emulsion and the phases are then analyzed for the solute. Care must be taken not to contaminate one phase with the other when sampling (R.T. Riley, personal communication). High performance liquid chromatography (HPLC) methods can also be used to determine the partition coefficient.

When determining the Log P for acids and bases pH is an important factor because the non-ionic form partitions more into the lipid phase. Also dimerization or trimerization may occur (4). The partition coefficient is calculated by taking the logarithm of the ratio of the concentrations in the octanol and water phases.

RATIONALE FOR CURRENT STUDY

The first phase of the study involved measuring the partition coefficients of compounds for which the partition coefficient is known in order to determine whether or not our methods were accurate. Two compounds with different partition coefficients were selected, ¹⁴C-benzoic acid and ¹⁴C-toluene. The second phase of the study involved determining the partition

coefficients for [^3H]PbTx-3, microcystin and lyngbyatoxin.

MATERIALS AND METHODS

^{14}C -benzoic Acid: The ^{14}C -benzoic acid (specific activity 21.8 mCi/mmol, NEN Research Products, Boston, MA) was reconstituted with 2 milliliters of distilled water. The stock solution was filtered with a 0.2 micron filter (Acrodisc, Gelman Sciences, Ann Arbor, MI). The concentration of the stock solution (1.495 $\mu\text{g}/\mu\text{l}$ or 12.25nmol/ μl) was determined using standard liquid scintillation (LSC) techniques and a Packard Model 1500 Tri-Carb Liquid Scintillation Analyzer, (Packard Instrument Co., Sterling, VA). A portion (40 μl , 59.82ng) of the stock solution was added to distilled water (total volume 100 μl) and placed in a polypropylene microcentrifuge tube with 100 μl of 1-octanol (Fisher Certified, Fisher Scientific Co., Norcross, GA). The determinations were done in quadruplicate. The tubes were shaken in a Roto-Torque heavy duty rotator (Cole Parmer, Chicago, IL) at room temperature (22°C) for 24 hours. The tubes were then centrifuged at approximately 1500 X G for 30 minutes to separate the phases. Aliquotes of each phase were analyzed with standard LSC techniques.

^{14}C -toluene: The [7- ^{14}C]-Toluene, (NEN Research Products) had a specific activity of 2.01×10^{-2} $\mu\text{Ci}/\text{mmol}$. ^{14}C -toluene (50 μl , 43.3 ng) was added to 1 ml of 1-octanol and partitioned into 5 ml of distilled water. A larger volume of water than octanol was required because most of the toluene would distribute into the octanol phase leaving little toluene in the water phase to be detected. The partition coefficient is dependent on the concen-

trations of the compound of interest in each phase, not the volumes of the phases. The partition coefficients were done in quadruplicate. The vials were shaken for 20 minutes at room temperature and then centrifuged for 30 minutes. Four ml of the water phase and 100 μ l of the octanol phase were analyzed by LSC in triplicate. Leo et al. have reported that as little as 5 minutes of gentle mixing are necessary to reach an equilibrium (4).

[³H]PbTx-3: A Waters Associates HPLC system (Waters division of Millepore, Milford, MA) was used to determine the radiochemical purity of both the stock solution of [³H]PbTx-3 and the [³H]PbTx-3 in the phases at the termination of the partition experiment. The HPLC detector was a Flo-One/Beta radioactive flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL). A Whatman Partisphere C₁₈ 5 micron cartridge column, (Whatman Inc., Clifton, NJ) was used. The mobile phase consisted of 85% methanol, 15% H₂O with a flow rate of 1 ml/min. The stock [⁴²-³H]PbTx-3 (radiochemical purity >99%, 9.93 Ci/mmol, mol. wt. 896; Dr. Baden, University of Miami, Miami, FL) was determined to have a concentration of 39 nmol/ml or 35.3 ng/ μ l. Stock [³H]PbTx-3 (883 ng, 25 μ l) was added to a vial, dried down under a stream of nitrogen and 1 ml each octanol and distilled water added. The vials were prepared in quadruplicate and were shaken at room temperature for 15 minutes. The vials were centrifuged for 15 minutes and each phase analyzed by LSC and HPLC. The HPLC analysis showed that PbTx-3 is unstable in 1-octanol (see Figure 1). Therefore, it was necessary to determine the partition coeffi-

cient for PbTx-3 in chloroform/water by using similar methods. HPLC analysis showed that PbTx-3 is stable in this system. Forty μ l (1412 ng) of the stock PbTx-3 was added to a 4 ml vial, dried down and 1 ml of chloroform (Fisher Certified) and distilled water added. Duplicate vials were shaken for 15 minutes and then centrifuged. Each phase was analyzed by LSC and HPLC techniques.

RESULTS AND DISCUSSION

The average of four measurements of the octanol/water partition coefficient ($\text{Log } P_{\text{octanol}}$) of ^{14}C -benzoic acid was 1.25 ± 0.06 (mean \pm standard error). The literature value for benzoic acid was 1.87, however, in the published method the pH was buffered to 7.4 and included hexadecylamine (4).

The experimentally determined the $\text{Log } P_{\text{octanol}}$ of ^{14}C -toluene to be 2.54 ± 0.05 . The literature gives values of 2.69, 2.73, 2.11 and 2.80 (4). On the basis of these results we decided that our method was accurate.

The $\text{log } P_{\text{octanol}}$ for $[^3\text{H}]\text{PbTx-3}$ was determined to be 2.20 ± 0.05 (mean \pm standard error, $n=2$). However, analysis of the phases using HPLC showed that the PbTx-3 was not stable in 1-octanol and began to degrade immediately. $[^3\text{H}]\text{PbTx-3}$ was stable in chloroform (CHCl_3) and the chloroform/water partition coefficient for PbTx-3 could be determined. The $\text{log } P_{\text{CHCl}_3}$ value was 2.20 ± 0.05 ($n=2$) (which was the same as the octanol/water partition coefficient). Leo and coworkers have derived linear equations relating the $\text{Log } P$ values in various solvents to the $\text{Log } P$ values in a reference system which is 1-octanol/water. They

group solutes into two general classes, hydrogen donors which include acids, phenols, alcohols etc. The other group includes hydrogen acceptors such as aromatic hydrocarbons, ethers, esters, ketones and others. The solvent chloroform is a weak H-donor and causes many of the solutes in the hydrogen donor group (e.g. barbiturates, alcohols, imides and amides among others) to be neutral in character (4). PbTx-3 is a polycyclic ether (see figure 2). This would place PbTx-3 in the hydrogen acceptor group. The linear equation for the CHCl_3 system with H-acceptor solutes is:

$$\text{Log } P_{\text{CHCl}_3} = 1.276 \text{ Log } P_{\text{Octanol}} + 0.171$$

Based on our data ($\text{Log } P_{\text{CHCl}_3} = 2.20$) the calculated $\text{Log } P_{\text{Octanol}}$ value is 1.59.

CONCLUSIONS

While the study was not intended to be in depth, it can be concluded that the partition coefficient for PbTx-3 probably lies in the range of 2.15 to 2.24 for CHCl_3 and to the best of our knowledge may be extrapolated to a value of 1.5 or 1.6 for octanol/water. Thus, PbTx-3 is roughly 160 times more soluble in chloroform than water and 32 times more soluble in 1-octanol than in water. Future plans include determining the partition coefficients for microcystin and lyngbyatoxin A.

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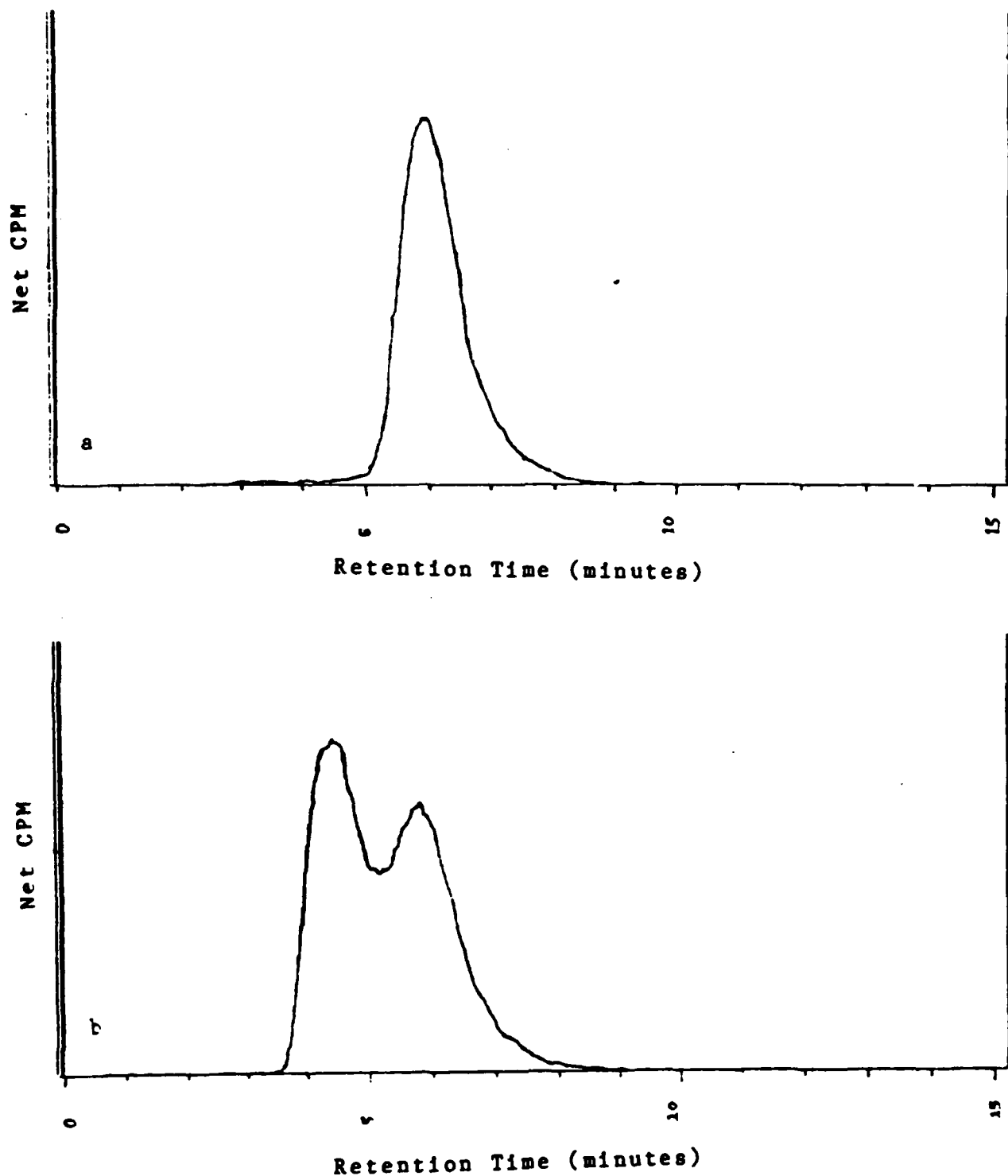
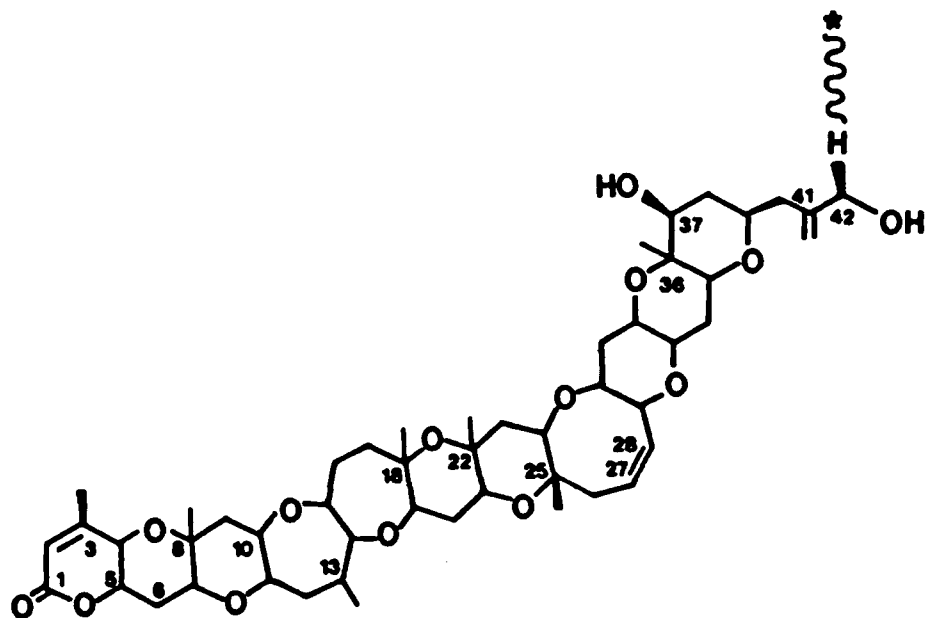


Figure 1. a) $[^3\text{H}]\text{PbTx-3}$ standard, Retention time= 5.97 min., 99.86% pure.
b) Chromatogram of PbTx-3 in octanol phase during determination of partition coefficient. The second peak is PbTx-3 , $R_t=5.8$ min., 51.31%. The first peak is the degradation product, $R_t=4.43$ min, 48.69%.



Brevetoxin
[42-³H] PbTx-3

Figure 2.

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